DOES NOT CIRCULATE

UNIVERSITY OF MICHIGAN

JOURNAL OF THE NATIONAL CANCER INSTITUTE

August 1956

Volume 17 • Number 2

A Publication of the
U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

JOURNAL OF THE NATIONAL CANCER INSTITUTE

JOHN R. HELLER, *Director*
National Cancer Institute

BOARD OF EDITORS

MICHAEL B. SHIMKIN, *Scientific Editor*

W. RAY BRYAN, *Associate Editor*
ELBERT A. PETERSON, *Associate Editor*
ELI M. NADEL, *Associate Editor*
EMMA SHELTON, *Associate Editor*
SEYMOUR H. WOLLMAN, *Associate Editor*

EDITORIAL STAFF

DORIS M. CHANEY, *Managing Editor*

ISABEL K. NEELY, *Asst. Managing Editor*
RENÉE T. COCHIN, *Assistant Editor*

U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE
BETHESDA 14, MARYLAND
UNITED STATES OF AMERICA

JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

UNIVERSITY
OF MICHIGAN

SEP 10 1956

MEDICAL
LIBRARY

Volume 17
Number 2

August 1956

The Histogenesis of Rous Sarcoma I Induced by Partially Purified Virus [1]

L. N. Loomis *and* A. W. Pratt,[2] *National Institute of Arthritis and Metabolic Diseases and National Cancer Institute,*[3] *Bethesda, Maryland*

The original work of Rous (*1,2*) on transplantation and induction of the Rous sarcoma and the subsequent studies of Mottram (*3*), Haddow (*4*), and Levine (*5*) on the nature of the Rous sarcoma dealt chiefly with implants of fresh tumor tissue, desiccated tissue, or filtrates of crude extracts of the tumor tissue. The earlier work was reviewed in the excellent article of Foulds (*6*) in 1934 on filterable tumors in the fowl.

The original view of Rous was that the sarcoma cells are derived by transformation of normal fibroblasts in the presence of the Rous agent into tumor cells, thus forming the subcutaneous tumor in the host. The recent work of Sanford *et al.* (*7*) indirectly supports this impression, since they found that the Rous agent survived only 11 to 14 days in tissue cultures of chicken monocytes, but it survived and multiplied for at least 6 months in cultures of chicken fibroblasts. Carrel concluded from his studies using tissue-culture techniques that the tumor cells of Rous sarcoma are derived from tissue macrophages (*8–14*). There is, however, no agreement with respect to the cell type that undergoes transformation into a tumor cell.

This paper is concerned primarily with the pathogenesis of the Rous sarcoma induced by "purified" virus. The development of more precise procedures by Bryan *et al.* (*15–17*) for isolating the virus offered an opportunity to study the problem of histogenesis of this tumor in the absence of extraneous factors.

The greater activity of the virus preparations was considered to be advantageous for study of early cell changes induced in the presence of virus because they were, in some measure, free of contaminating substances which could lead to tissue reactions that might mask the earliest cell changes. While it is recognized that it is difficult to determine the cellular origin of the tumor cell of the Rous sarcoma, an attempt has been made to correlate chronologically the morphologic alterations induced in the

---

[1] Received for publication December 21, 1955.

[2] We wish to thank Dr. W. Ray Bryan for supplying the partially purified Rous virus and Dr. George L. Fite for his many helpful discussions.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

presence of the virus in normal tissue components—specifically the fibroblast—with the appearance of the tumor cells.

Materials and Methods

Rous sarcoma virus (*agent of chicken tumor I*).—Partially purified cell-free virus suspensions were of 2 types, designated herein as I and II. Type I preparations were total-microsome fractions that had been separated from Rous tumor-tissue extracts by differential centrifugation. The technique employed was similar to that previously described by Bryan *et al.* (*15,16*). The sedimented fractions were resuspended in citrate buffer (0.05 *M* sodium citrate, *p*H 6.7) in a final concentration equivalent to 1.5 gm. of tumor tissue per cc. This material was diluted 1:100 before injection with 0.05 *M* citrate buffer containing 2 percent normal horse serum. Type II preparations showed "greater activity." Separation of the virus from the total-microsome fraction was done according to procedures described by Bryan, Moloney, and Calnan for their material CT 522 (*17*). For use in these studies, type II virus suspensions were freshly prepared in all instances except lot CT 522, which was a frozen standard (see *17*). For injection into experimental chicks, the concentrated suspensions were diluted 1:100 with 0.05 *M* citrate buffer. Horse serum was eliminated as the diluent for all type II preparations.

Test chickens (*table 1*).—Over 700 New Hampshire Red and Rhode Island Red chickens were used, all being 5 weeks old at the time of inoculation. They were obtained as day-old chicks (equal number of males and females) from a local commercial hatchery. The chicks were raised in a commercial-type brooder to 3 weeks of age, then transferred to a commercial battery brooder with a 200-bird capacity. All birds were fed dry Chick Startena (Purina Co.) and water on demand.

Inoculations.—The volume of all inocula was 0.2 ml. and the site of injection was the ventral subcutaneous tissue of the right wing. In the first 2 groups, injections were made into the subcutaneous area after passing the needle through the M. extensor carpi radialis. In the groups in which it was desired to study the invasion of muscle tissue by tumor cells, trauma and direct inoculation of the muscle tissue were avoided by passing the needle anterior to the muscle through the skin.

Histologic procedures.—The entire soft-tissue area of the wing between the humeral and carpal joints was removed, and either the whole was fixed or it was divided into small pieces. The fixatives used were: Zenker's fluid, buffered 10 percent formalin, Bouin's fluid, and 95 percent ethyl alcohol (for mucicarmine stains). The stains used were: Mayer's acid-alum-hematoxylin and eosin Y; Reinhardt-Abul-Haj variant of Hale's method for mucin ("acid mucopolysaccharide"); Mayer's mucicarmine stain, Masson's trichrome, acid-phosphatase (Gömöri), periodic acid-Schiff reaction, Parson's stain (methylene blue and ethyl eosin for Negri bodies) and azure A-eosin B (*18*).

The total of 633 birds injected with Rous virus was studied histologically.

TABLE 1.—*Twelve groups of birds were inoculated, as follows:*

Group	Material injected	Number of birds	Hours after injection*	Procedure
1	Rous sarcoma virus (type I).	95	24	9 birds killed daily for 10 consecutive days. 5 birds killed on 12th day.
2	Rous sarcoma virus (type I).	50	48	10 birds killed daily for 5 consecutive days.
3	Control group—NON-INJECTED.	12	—	Specimens taken from comparable wing area.
4	Rous sarcoma virus (type II).	50	24	10 birds killed daily for 4 consecutive days. 10 birds killed on the 8th day.
5	2% Horse serum in 0.05 *M* citrate buffer (*p*H 6.7).	25	48	5 birds killed daily for 5 consecutive days.
6	Rous sarcoma virus (type I).	50	48	10 birds killed daily for 5 consecutive days.
7	Repeat of group 6			
8	Repeat of group 6			
9	Rous sarcoma virus (type II) (lot 495).	80	24	10 birds killed daily for 7 consecutive days. 10 birds killed on 12th day.
10	Rous sarcoma virus (type II) (lot 495).	200	48	10 birds killed daily for 10 consecutive days (Zenker's fixative). 5 birds killed daily for 10 consecutive days (buffered formalin). 5 birds killed daily for 10 consecutive days (Bouin's fixative). (Total of 20 birds killed daily for 10 consecutive days.)
11	Rous sarcoma virus (type II) (lot 522).	36	72	6 birds killed daily for 6 consecutive days—fixed in 95% ethyl alcohol (mucicarmine stain).
11a	Control group for group 11—NON-INJECTED.	36	72	Procedure same as in group 11.
12	Rous sarcoma virus (type II) (lot 522).	40	72	2 birds killed daily for 10 consecutive days—fixed in 60% ethyl alcohol. 2 birds killed daily for 10 consecutive days—fixed in reagent grade acetone (alkaline-acid phosphatase stains).

*This column refers to the time interval between inoculation of the group and the initial sacrifice.

The tissues of 61 normal chickens, and of chickens injected with citrate buffer-horse serum without virus, were also studied as controls. Occasionally, serial sections were made of the tumor-bearing tissues at an early stage following inoculation of the virus.

The remainder of the birds were observed until death, usually at about 35 to 40 days.[4] Approximately 1 percent of the tumors regressed.

Results

Gross Observations on Living Birds and Sites of Inoculation

The active Rous sarcoma virus rapidly produced gross manifestations of the tumor process. Congestion and thickening appeared at the site of inoculation within 3 to 4 days, and translucent blisters (0.5 × 2.0 mm.), by the 4th day; by the 5th day the rapidly expanding growth had developed more numerous and larger vesicles containing mucin. Bluish-purple discoloration (ecchymosis) occurred in nearly 50 percent of birds with tumor by the 10th day. Between the 14th and 20th days the tumor and the entire ventral surface of the wing often displayed a diffuse greenish pigmentation; ulceration of the larger tumors was frequent; many became secondarily infected; most of the tumors penetrated through to the dorsal surface of the wing. Feather development over the tumor site ceased, and only a few stunted feathers remained. Within 10 days the subjacent muscle was involved by tumor tissue. The appearance of wing-drop varied from bird to bird, depending on the amount of muscle involvement; it was obvious by the 15th day, and progressed until the large wing feathers were touching the floor about the 24th day. Eighteen to 20 days after inoculation, tumor-bearing birds developed cachexia and progressive weakness; the earliest deaths occurred at 18 days, and a sharp rise in mortality was noted between 20 and 30 days, reaching 95 percent of inoculated birds by the 35th day.

Microscopic Observations

In the normal subcutaneous tissue of the fowl (fig. 1), many of the cells are fibroblasts (fibrocytes) which have oval nuclei containing numerous discrete, relatively uniform chromatin granules, plus 1 or more nucleoli. These cells show bipolar orientation into long, parallel rows and are associated with collagen fibers. This type of connective tissue can be seen in the dermis, particularly its deepest border, in the subcutaneous area as nearly parallel strands beneath the dermis, and as a dense band forming the cutaneous surface of the skeletal-muscle fascia which varies in collagen content, thickness, and density with its location.

Forty-eight hours after inoculation of the virus suspension (fig. 2), the picture of subcutaneous cellular alteration can be seen involving the fibroblasts in all 3 of the areas described in the normal tissue. The fibroblasts become more distinct as individual cells because of increased size and basophilia and give the appearance of chains of swollen cells. The nucleus of many of these cells shows a more distinct nuclear membrane with a cleared nucleoplasm and an enlarged nucleolus (or inclusion).

At 72 hours (fig. 3), and at 96 hours (fig. 4), these basophilic, enlarged

[4] No spontaneous transfer of tumors was observed in more than 200 control birds kept in the battery of cages with the tumor-bearing birds.

cells appeared to be neoplastic. Some of them lay free in the tissue spaces, while many remained attached to each other and to connective-tissue elements. The pattern of nuclear chromatin material appeared altered in nearly all cells (fig. 5). The changes were characterized by margination of the chromatin and by the appearance of 1 or more much larger nucleoli than normally seen, but there was little other dispersed chromatin material. These cells showed cleavage from tissue matrix and became greatly enlarged; the nuclei were swollen and displayed large, eosinophilic, dense, refractile intranuclear structures which frequently seemed separated from the nuclear membrane by a clear halo. The nature of these intranuclear inclusions is unknown but they could be simply altered nucleoli.

In the dense subcutaneous connective tissue, the polar orientation of fibroblasts into long, parallel rows lying adjacent to collagen fibers was conspicuous (fig. 11). Trichrome stains (Masson's) illustrated the early altered fibroblasts within dense, collagenous connective tissue, still with intact polarity but greatly enlarged and loosely held (fig. 12).

Definite changes were noted in the rows of cells which remained attached to the deeper margin of the dermis. The nuclei of most of these cells showed margination of chromatin and a single enlarged nucleolus and/or inclusion. These rows of transformed cells appeared to cleave from the subcutaneous matrix, their continuity broken by splitting between cells, with the appearance of freed, discrete tumor cells lying in loose, fibrillary tissue (fig. 7). The resulting picture is that of a spindle-cell tumor of the classical Rous sarcoma variety (figs. 8 and 9).

By the 5th day (fig. 6), the tumor cells formed aggregates of tissue, producing elevated masses palpable in the living bird. The tumor contained small amounts of mucin. Perivascular clumps of macrophages containing tissue debris were found in the tumor area.

The cytologic pattern of the tumor became characteristic by the 6th day. Many proliferating spindle-shaped tumor cells were at the periphery of the lesion, while frequently the basophilic round tumor cells were more numerous in the center. The subcutaneous spread of the tumor by contiguous growth progressed rapidly, for in the same period the tumor cells had invaded the margins of the underlying skeletal muscle.

From the 6th day on, the rapidly increasing volume of the tumor was primarily due to massive production of mucin, demonstrable by special stains (Hale's or Mayer's mucicarmine methods). The extracellular mucin was associated with both the spindle-shaped and the basophilic round-cell forms of the tumor. Macrophages were found in the areas of mucin within the tumor.

A lymphocytic response was associated with the development of this tumor. It was apparent at 48 and 72 hours, and reached a maximum degree on the 5th or 6th day as perivascular infiltrations, lymphoid plaques, and dense aggregations of lymphocytes at the periphery of the expanding tumor. After the 6th day, lymphocytes became progressively less in number and ultimately disappeared.

Histochemical procedures proved illuminating. Divided samples of tumor obtained from lesions which were 72 hours to 20 days post inoculation, were fixed in alcohol and in acetone and stained by Masson's trichrome and acid phosphatase, respectively. Weiss and Fawcett (*19*) stated that chicken macrophages in tissue culture were strongly acid-phosphatase positive. In this study, 8-day Rous tumor tissue itself was negative for acid phosphatase at 6 hours' incubation and slightly less positive than skeletal-muscle tissue at 24 hours' incubation. An exception to this was noticed when Rous tumor tissue was close to skin or about blood vessels where it gave a stronger reaction. Macrophages in these areas were much more strongly positive, appearing brownish-black. Periodic acid-Schiff-positive granules were evident in the cytoplasm of 8-day Rous tumor cells.

These results with acid-phosphatase stain and also the periodic acid-Schiff-positive granules in the Rous tumor-cell cytoplasm are similar to those reported by Cater (*20*).

Toluidine-blue stains of 168- and 192-hour Rous tumor tissue demonstrated metachromatic material in tumor mucin. This metachromatic material was removed by 2-hour hyaluronidase incubation. The mucin is considered to be a chondroitin sulfate mucin.[5]

The "inclusion bodies" of the Rous tumor-cell nuclei are either acidophilic or basophilic according to the *p*H of the stain buffers, but a balanced azure-eosin stain (where skeletal muscle is stained a clear acidophilic pink while the muscle-cell nuclei stain a deep blue-black) reveals the "inclusion bodies" as amphophilic. The "inclusion bodies" were not acid-fast; nor were they acidophilic after Parson's stain.

Subsequent to the establishment of the characteristic tumor pattern as described above, basophilic large round cells appeared in the center of the lesion, while the spindle cells were more evident at the periphery. By the 7th or 8th day, the subcutaneous area was predominantly infiltrated by the former type, while at the margin of the tumor (invading muscle) spindle cells were more abundant. The basophilic cell forms were also ultimately found in invaded muscle tissue, but only in more advanced lesions; they seemed to originate by continued shortening of the spindle-cell forms, associated with a progressive increase in nuclear size and increased cytoplasmic basophilia (*cf.* description of Doljanski and Tenenbaum for tissue-culture fibroblasts, *21,22*). The nuclear alterations were of the same nature as seen in the spindle cells, but cytologic details were often obscured by the intense basophilia (fig. 10).

Mucin production has a characteristic early pattern of association with the tumor cells. The role of the mucin is not understood, even though it progressively occupies an increasing volume of the tumor. The lymphocytic-cell and macrophage-cell response appears to be an inflammatory reaction of the host.

---

[5] R. D. Lillie, personal communication.

Discussion

A large number of chickens receiving standard inocula of partially purified Rous sarcoma virus developed reproducible gross lesions and microscopic cellular alterations which represented tumor formation.

This study reveals that within 48 hours following inoculation of the Rous virus, rows of altered subcutaneous fibroblasts may be clearly identified. The intranuclear alterations consisted of margination of chromatin and appearance of large, dense, eosinophilic (hematoxylin and eosin stain), refractile intranuclear bodies which frequently seemed separated from the nuclear membrane by a clear halo. These structures meet the morphologic requirements of an intranuclear inclusion body but their exact nature is unknown. The initial nuclear alterations are followed by progressive swelling and eventual loss of polarity, with subsequent escape into the subcutaneous spaces as free cells which, in our opinion, are the tumor cells of Rous sarcoma. The subsequent alteration in the form of these cells, while free in the subcutaneous space, may be considered to be due to some as yet unknown physicochemical environmental change.

These studies involve the correlation of observations made on different lesions obtained as successive stages of the formation of a tumor by serial killing of the hosts. It is felt that a valid correlation can be obtained in this manner, since the procedures involved in these studies, and the gross and microscopic growth characteristics of the induced tumors in the groups of chickens used, are reproducible.

The actual process of transformation of the fibroblasts into tumor cells cannot be demonstrated in this material. However, the material studied in chronological order showed the disappearance of altered fibroblasts with the almost concomitant appearance of characteristic early tumor cells, thus indicating that the subcutaneous fibroblast is the cellular component of normal tissue from which the tumor cell of Rous sarcoma is derived.

Summary

Formation of Rous sarcoma has been studied in a large number of chickens that received inocula of partially purified Rous virus. Rows of altered subcutaneous fibroblasts may be identified within 48 to 72 hours after the virus is injected. The intranuclear alterations have been described.

With release of the altered cells into the subcutaneous tissue, 2 types of tumor cells appear—1) a spindle-cell form from which 2) the basophilic round-cell form appears to be derived.

The tumor cells contained little acid phosphatase, while the macrophages, found in their usual role of phagocytes, are rich in acid phosphatase. The tumor mucin is considered to be a chondroitin sulfate mucin.

This study indicates that the Rous sarcoma tumor cell is derived from the normal subcutaneous fibroblast in the presence of Rous virus.

383538—56——2

References

(*1*) Rous, P.: A transmissible avian neoplasm. J. Exper. Med. 12: 696–705, 1910.

(*2*) ———: A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exper. Med. 13: 397–411, 1911.

(*3*) Mottram, J. C.: Effect of radiation on Rous chicken tumour. Brit. J. Exper. Path. 9: 147–154, 1928.

(*4*) Haddow, A.: The application of vital staining to the histogenesis of the Rous sarcoma I. J. Path. & Bact. 37: 149–155, 1933.

(*5*) Levine, M.: The cytology of the tumor cell in the Rous chicken sarcoma. Am. J. Cancer 36: 276–302, 386–430, 581–602, 1939; 37: 69–107, 1939.

(*6*) Foulds, L.: The Filterable Tumours of Fowls: A Critical Review. Imp. Cancer Res. Fund, London, 1934.

(*7*) Sanford, K. K., Likely, G. D., Bryan, W. R., and Earle, W. R.: The infection of cells in tissue culture with Rous sarcoma virus. J. Nat. Cancer Inst. 12: 1317–1343, 1952.

(*8*) Carrel, A.: Cultures pures de fibroblastes provenant de sarcomes fusocellulaires. Compt. rend. Soc. biol. 90: 1380–1382, 1924.

(*9*) ———: La malignité des cultures pures de monocytes du sarcome de Rous. Compt. rend. Soc. biol. 91: 1067–1069, 1924.

(*10*) ———: Action de l'extrait filtré du sarcome de Rous sur les macrophages du sang. Compt. rend. Soc. biol. 91: 1069–1071, 1924.

(*11*) ———: Effets de l'extrait de sarcomes fusocellulaires sur des cultures pures de fibroblastes. Compt. rend. Soc. biol. 92: 477–479, 1925.

(*12*) ———: Essential characteristics of a malignant cell. J.A.M.A. 84: 157–158, 1925.

(*13*) ———: Some conditions of the reproduction in vitro of the Rous virus. J. Exper. Med. 43: 647–668, 1926.

(*14*) Carrel, A., and Ebeling, A. H.: The transformation of monocytes into fibroblasts through the action of Rous virus. J. Exper. Med. 43: 461–468, 1926.

(*15*) Bryan, W. R., Maver, M. E., Moloney, J. B., Thomas, M. A., and Sells, M. T.: Comparative stability of the agent of chicken tumor I in phosphate and borate buffers during incubation for 3 hours at 37° C. J. Nat. Cancer Inst. 10: 647–656, 1949.

(*16*) Bryan, W. R., Lorenz, E., and Calnan, D.: Protecting action of citrate on the agent of chicken tumor I (Rous sarcoma virus) during roentgen radiation *in vitro*. J. Nat. Cancer Inst. 13: 1447–1461, 1953.

(*17*) Bryan, W. R., Moloney, J. B., and Calnan, D.: Stable standard preparations of the Rous sarcoma virus preserved by freezing and storage at low temperatures. J. Nat. Cancer Inst. 15: 315–329, 1954.

(*18*) Lillie, R. D.: Histopathologic Technic and Practical Histochemistry. New York, Blakiston Company, 1954.

(*19*) Weiss, L. P., and Fawcett, D. W.: Cytochemical observations on chicken monocytes, macrophages and giant cells in tissue culture. J. Histochem. & Cytochem. 1: 47–65, 1953.

(*20*) Cater, D. B.: A histochemical and biochemical study of some effects produced by the Rous sarcoma upon the endocrine organs of cocks and hens. J. Path. & Bact. 63: 269–284, 1951.

(*21*) Doljanski, L., and Tenenbaum, E.: Studies on Rous sarcoma cells cultivated in vitro. I. Cellular composition of pure cultures of Rous sarcoma cells. Cancer Res. 2: 776–785, 1942.

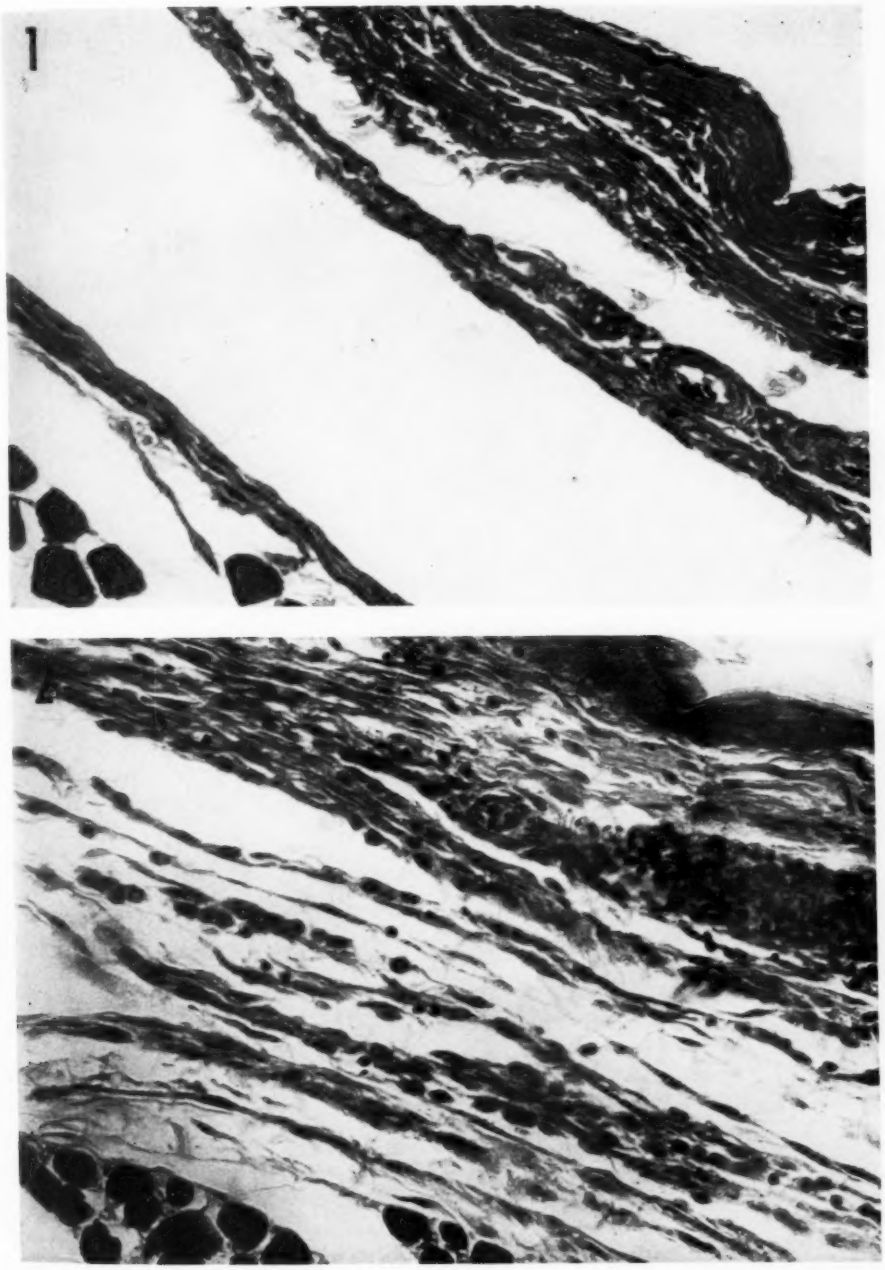
(*22*) Tenenbaum, E., and Doljanski, L.: *Ibid.* II. Morphologic properties of Rous sarcoma cells. Cancer Res. 3: 585–603, 1943.

PLATES

PLATE 11

FIGURE 1.—An area of skin and subcutaneous tissue representative of the normal structures that were exposed to the Rous sarcoma virus. Hematoxylin and eosin. × 455

FIGURE 2.—An area similar to that of figure 1, 48 hours after inoculation of 0.2 ml. of partially purified Rous sarcoma I virus. Hematoxylin and eosin. × 455

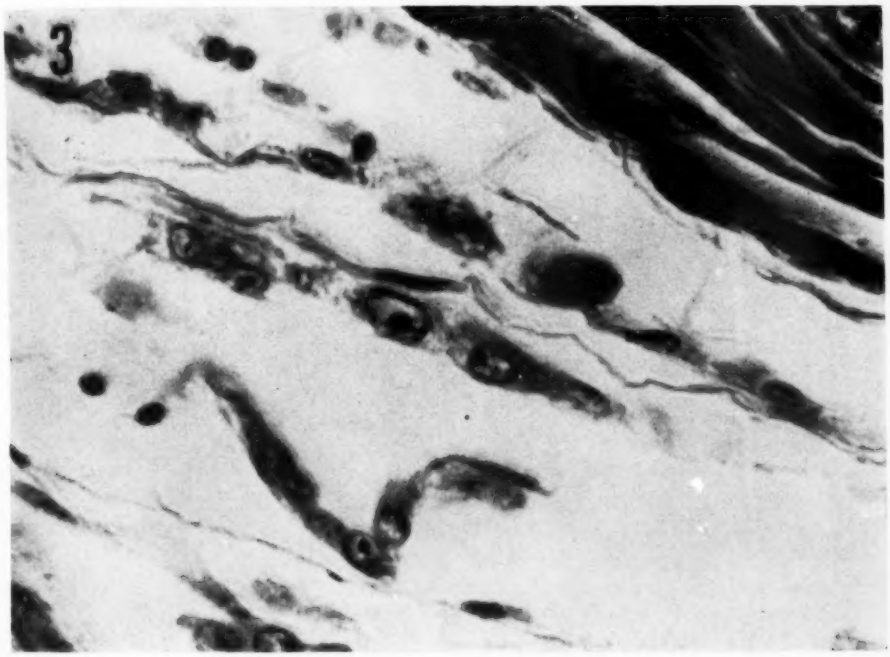


383538—56——3

PLATE 12

FIGURE 3.—Subcutaneous area, chicken. Showing typical changes in fibroblasts at 72 hours; margination of chromatin and enlarged nucleoli; and characteristic basophilism of a transformed rounded fibroblast. Hematoxylin and eosin. × 950

FIGURE 4.—Subcutaneous area, 96 hours after inoculation. Swollen fibroblasts forming cords extend in rows from loose tissue parallel to skin surface; no invasion of muscle. Hematoxylin and eosin. × 320


3

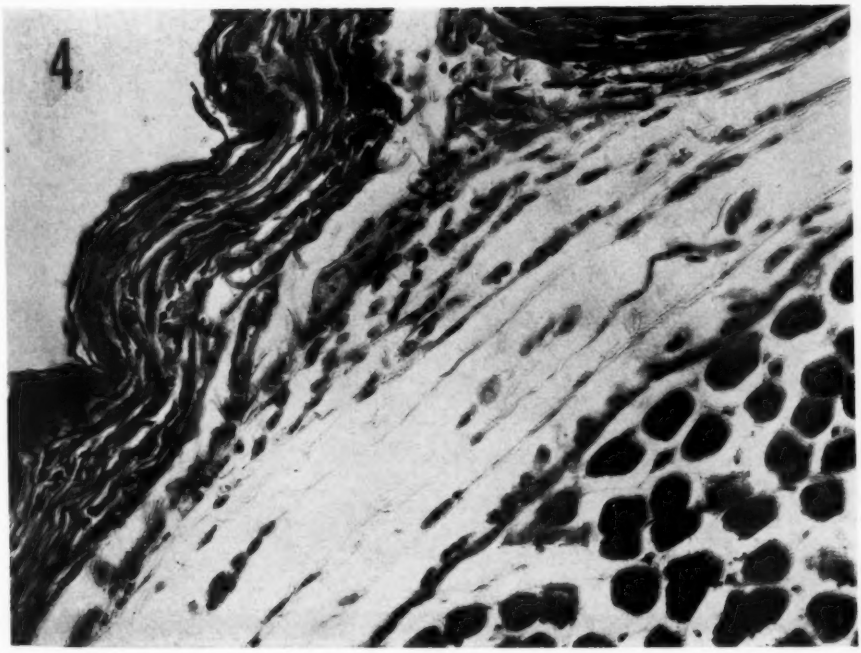

4

PLATE 13

FIGURE 5.—Subdermal area at 96 hours. Showing the alterations from (*a*) fibrocytes with their nuclear alterations, (*b*) free malignant spindle forms, and (*c*) basophilic rounded forms. Hematoxylin and eosin. × 800

FIGURE 6.—Subcutaneous area, 5 days after inoculation with the Rous virus. Tumor cells arranged as sheets beneath the dermis; the long horizontal connective-tissue strands are disrupted by the tumor cells breaking away from parent-site cells; minor degree of lymphocytic infiltration. Hematoxylin and eosin. × 210

5
b
a
c

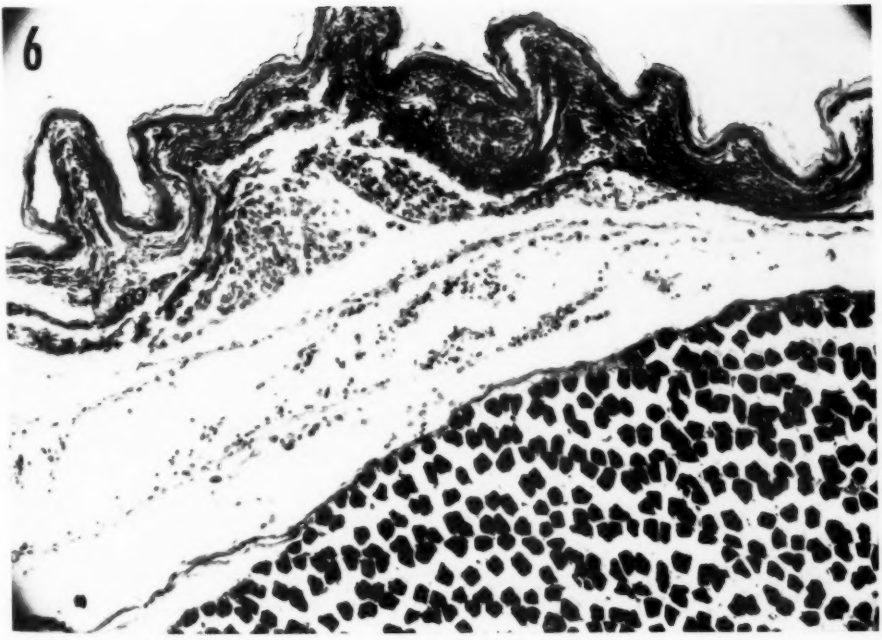
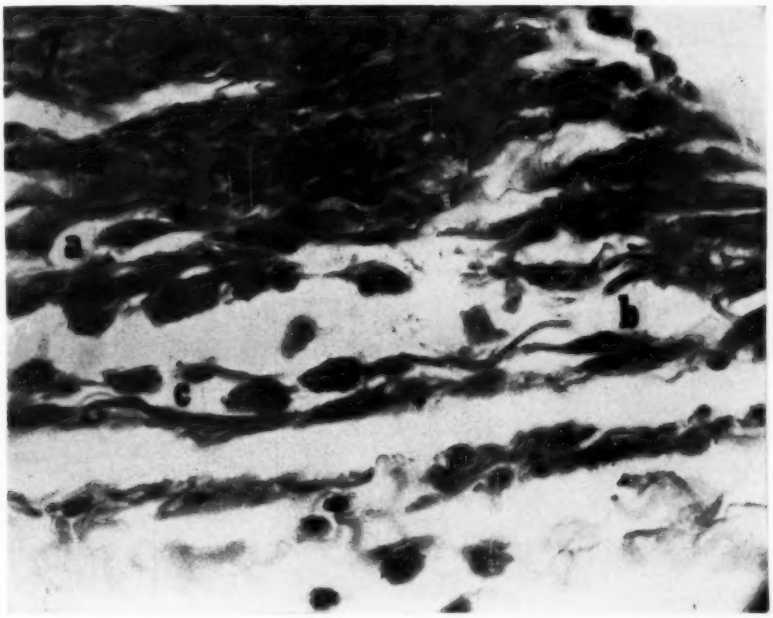

6

PLATE 14

FIGURE 7.—Rous sarcoma, chicken. Illustrates an area of dense connective tissue undergoing malignant alteration. (*a*) Enlargement and the accompanying separation of fibroblasts as malignant spindle forms, (*b*) free, well-defined fibroblastic forms, (*c*) the continued enlargement and shortening resulting in the basophilic round forms. Hematoxylin and eosin. × 800

FIGURE 8.—Illustrates early tumor formation at a secondary area of the subcutaneous tissue; the 5-day-old larger primary area is in another field of the section. The secondary area has the appearance of a 72- to 96-hour-old Rous tumor; notice presence of both fibroblastic and basophilic round-cell types; the loss of collagenous material is typical. Hematoxylin and eosin. × 500


a
b
c

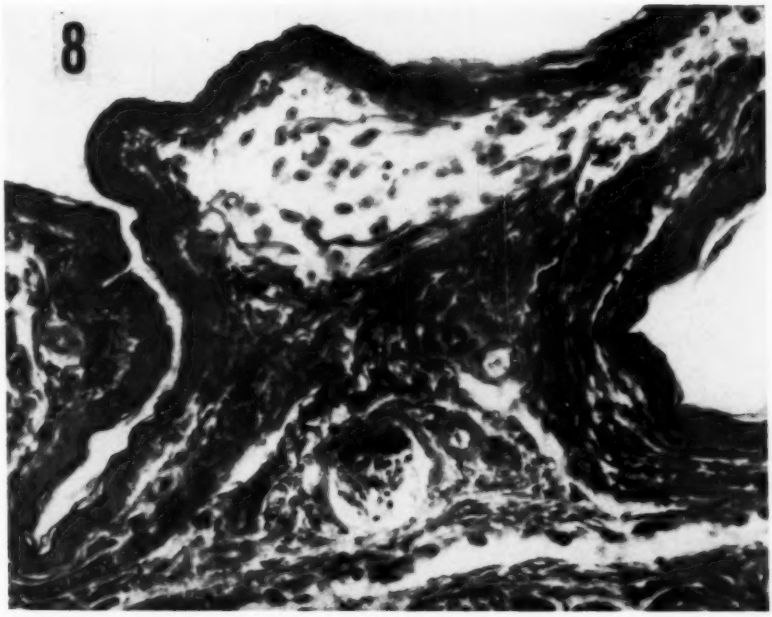
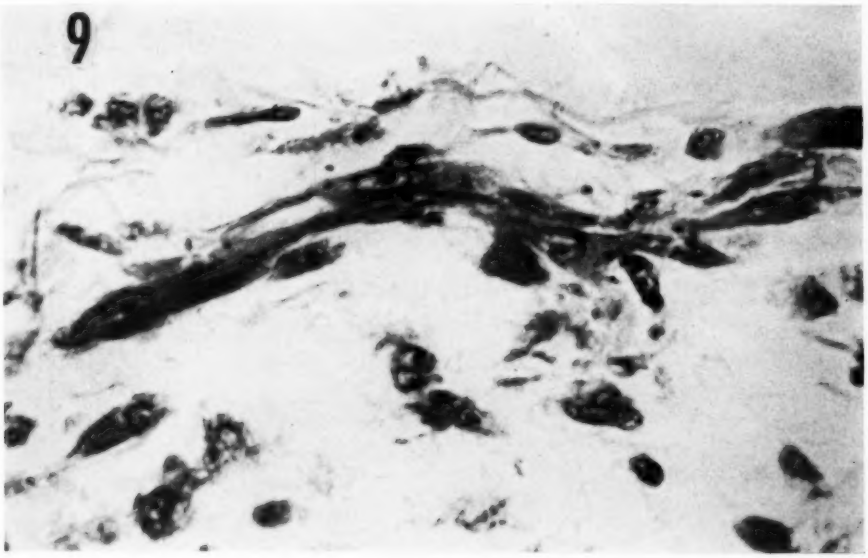
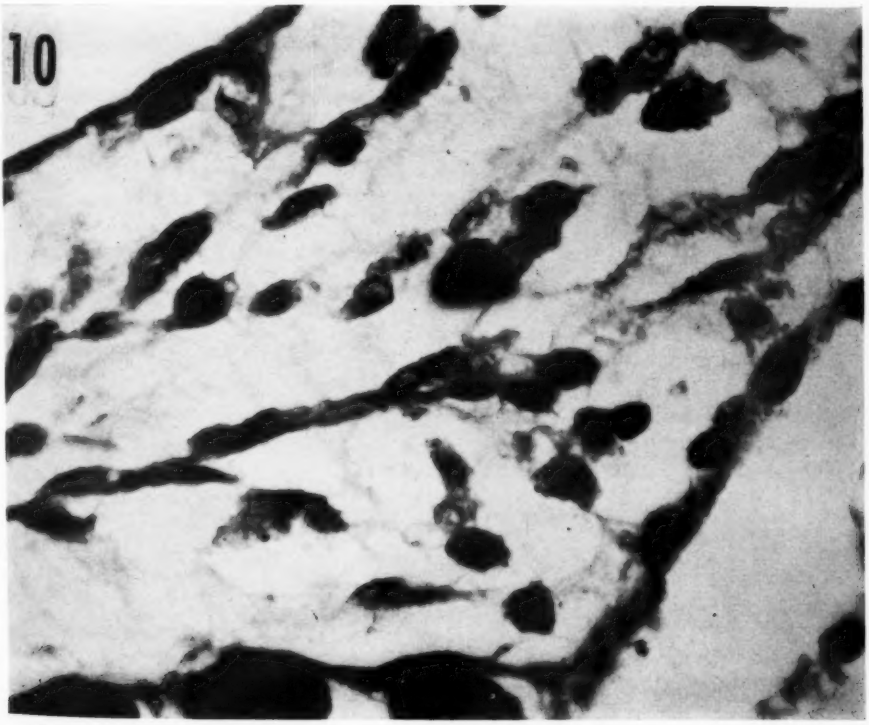

8

PLATE 15

FIGURE 9.—Rous tumor cells of fibroblastic (spindle) type at 168 hours. Seen at the periphery of tumors that are rapidly expanding and within early muscle lesions; a few shortened and rounded forms are also present. Hematoxylin and eosin. × 810

FIGURE 10.—Rous tumor cells in an area of connective tissue overlying muscle bundles. As the tissue loses its cohesion the released fibroblasts swell and shorten into rounded forms. Hematoxylin and eosin. × 800





383538—56——4

PLATE 16

FIGURE 11.—Normal, dense, collagenous connective tissue over skeletal-muscle area. Masson's trichrome stain. × 285

FIGURE 12.—An area comparable to figure 1 at the periphery of an 8-day Rous tumor, showing the area and stages of fibroblastic transformation. Notice the disruption of polar orientation, the early mucinous material, and the "intranuclear inclusions." Masson's trichrome stain. × 285

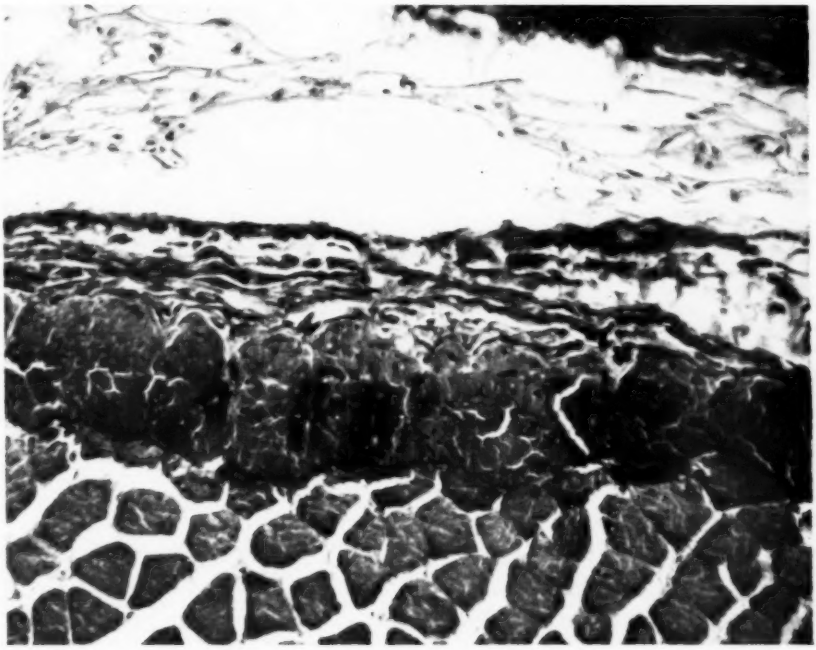


FIGURE 11.

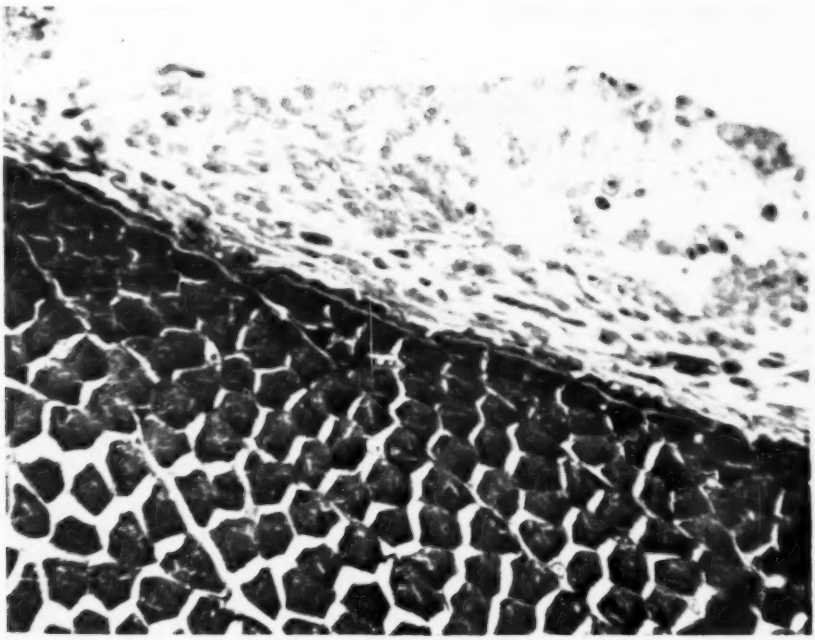


FIGURE 12.

Loomis and Pratt

Transplantability of a Canine Thyroid Carcinoma through Thirty Generations in Mixed-Breed Puppies [1, 2]

M. W. Allam, L. S. Lombard,[3] E. L. Stubbs, *and* J. F. Shirer, *School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

The transplantation of a spontaneous canine thyroid carcinoma through 6 generations of mixed-breed puppies was previously reported by Allam *et al.* (*1*). This was accomplished with the use of agents to alter the natural resistance after unsuccessful attempts to transplant 13 spontaneous canine tumors to normal dogs. X radiation or nitrogen mustard was used to induce tumor growth.

The following report describes the 7th through the 30th transplant series in mixed-breed puppies, utilizing the same techniques with minor modifications.

Materials and Methods

From earlier data, total-body X irradiation appeared to be the best method of preparing the tissues of the canine for accepting the transplant.

The previous article (*1*) described total-body X-irradiation doses of 300 to 500 r, but losses from 500 r seemed high, so that in later series only 300 r were used. The experiments here reported were performed with a reduced radiation exposure of 150 to 300 r. A high percentage of transplant growths occurred at this reduced level. Cortisone or hydrocortisone [4] was not used beyond the 11th generation.

The tumor transfer material in the previous experiments was minced in physiologic saline or Locke's solution. The present experiments were made with tumor transfer material minced in 5.3 percent dextrose solution, since Craigie (*2*) has shown that such a medium conserves a high percentage of viable tumor cells. The medium was kept cold (4° C.) during the mincing and until the transplants could be made. A 10-gauge needle attached to a metal syringe with a glass barrel was used to withdraw the tumor tissue from the mincing dish and to transplant it into the recipient host. Approximately 2 cc. of the suspension of tumor cells was injected into each site. The transplants were made subcutaneously in the right and left flanks of most of the dogs. Intramuscular injections were made into the right thigh muscle in a small group of puppies.

[1] Received for publication December 13, 1955.

[2] This investigation is supported by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] *Present address:* National Cancer Institute, Bethesda 14, Md.

[4] Nitrogen mustard (Mustargen, Merck), cortisone (Cortone, Merck), and hydrocortisone (Hydrocortone, Merck) were supplied by courtesy of Merck and Company, Rahway, N. J.

Throughout the course of the experiment, deaths from unrelated causes were frequent. It is felt that the irradiation intensities used here, in addition to being innately injurious to the puppies, also predisposed them to severe intercurrent infections. Pneumonitis and lesions of acute viral entities of dogs were frequently noted at the necropsy table. It is probable that the animals surviving less than 7 days, when considered as a group, do not present a valid criterion for transplant growth or nongrowth. Thus, they are discarded in transplant-growth determinations and in calculation of "take-rate" percentages.

Because of the difficulty inherent in determining the presence or absence of tiny rests of viable tumor cells in large muscle masses or in the peritoneal cavity, complete regression is here considered a valid determination only in the subcutaneous area.

Results and Discussion

Irradiated Animals

For the 7th to the 30th transplant series inclusive, 372 mongrel puppies were X-irradiated and injected with tumor cells in various sites. Among 324 animals living 7 days or longer, 278 (85.8%) developed grossly recognizable tumors. The latent period (injection to gross recognition, in days) varied slightly among the inoculation sites. Metastases were occasionally observed in this series; they were not found earlier than 16 days after injection. The most frequent metastatic site was the regional lymph gland. Distant lymph nodes and lungs were involved in 2 animals of the subcutaneous group at 38 and 48 days, respectively. Although other puppies lived longer than 48 days, involvement of sites other than the regional nodes was not seen in X-irradiated animals.

The gross characteristics of the tumor were similar to those described previously (*1*). Growth of the transplanted-tumor mass generally proceeded uneventfully until about the 14th post-inoculation day; cut surface at this time revealed homogeneous, glossy, gray-pink tumor tissue. Although the average tumor reached even greater gross proportions by the 21st post-inoculation day (8.5 × 5.5 × 5.0 to 12.0 × 10.5 × 6.5 cm.—fig. 1), the latter increase in size could be explained to a great extent on the basis of scattered areas of hemorrhage and necrosis within the central portions of the tumor mass. Ulceration occurred occasionally during the 4th to the 5th week. Lymph nodes containing metastatic tumor cells were markedly enlarged, friable, and dark bluish-red (fig. 2).

Microscopically, the tumor resembled the original and the first 6 transplant series, with the exception of giant cells, which were absent in advanced generations. The tumor cells were fairly uniform in size, and each contained a large, vesicular nucleus with a prominent nucleolus. The cytoplasm was scant, and stained poorly with hematoxylin and eosin. The tumor contained numerous capillaries during the period of growth

extending from 7 to 14 days following transplantation (fig. 3). By the 21st day, many areas of hemorrhage and small, scattered areas of necrotic and degenerating cells were seen. Beginning with approximately the 28th day, large areas of necrotic and degenerating tumor cells occupied the center of the nodule. The tumor cells surrounding the central necrotic area were smaller and contained hyperchromatic nuclei. In tumors present 30 to 40 days or longer, central necrosis was a prominent gross and histologic feature. In many of these, a few viable neoplastic cells were noted in close proximity to blood vessels which traversed otherwise completely necrotic tissue. The peripheral zone of the tumor nodule usually consisted of healthy-appearing cells, with numerous mitotic figures. In lymph-node metastases, carcinoma cells were observed within the sinusoids as well as throughout the stroma of the gland (fig. 4).

Three hundred and thirty-six irradiated animals received injections of tumor cells in the subcutis of the flank; 291 lived 7 days or longer, and of the latter, 254 (87.3%) developed grossly recognizable tumors. In most of these puppies, a poorly defined swelling was apparent by the 5th post-inoculation day; by the 7th day (var. 5th to 9th), a firm, discrete nodule appeared.

Inguinal lymph glands were the most frequent site of metastasis in this subcutaneous group (16 dogs). Additional dissemination of neoplastic cells to iliac nodes occurred in 12 instances. Two puppies showed generalized metastatic lesions. Thirty-eight complete regressions were noted; these occurred, generally, from 3 to 6 weeks after inoculation.

Seventeen irradiated animals received minced tumor tissue intramuscularly in the right thigh; of 14 that lived 7 days or longer, 10 (71.4%) developed a grossly recognizable tumor. Growth was not generally recognized until approximately the 14th day after transplantation, partially because of the diffuse thickening of the thigh. Two animals that died at 21 and 35 days, respectively, had inguinal-node involvement only.

Macroscopically, the tumor infiltrated the muscle in a diffuse manner. Microscopically, scattered areas of hemorrhage and necrosis were seen in the specimens taken at necropsy on the 21st and 25th post-inoculation days. The tumor cells were histologically similar to those described previously.

Animals Pretreated with Nitrogen Mustard

Eighteen (69.2%) of 26 animals pretreated with nitrogen mustard and receiving subcutaneous flank inoculations developed tumors which were recognizable by the 7th or 8th day. Although 16 puppies lived 16 days or longer, metastases were not seen in this group. Regressions were fairly frequent, occurring in 9 dogs. Gross and histologic characteristics were similar to those of the subcutaneous tumors in X-irradiated animals.

Untreated Animals

Of 64 dogs receiving tumor tissue either subcutaneously or intramuscularly and living longer than 6 days, 39 (60.9%) developed grossly recognizable tumors.

TABLE 1.—*Growth of transplants in pretreated and untreated dogs**

Pretreatment and generation No.	Injection site	No. of dogs injected	No. of dogs living 7 + days	No. of dogs developing tumors	Tumor growth (*percent*)	No. of dogs living 16 + days	Metastases		No. of dogs with complete regression
							No. of dogs	Site	
X irradiation									
7–30 inclusive	SC	336	291	254	87. 3	138	4 12 2	Inguinal Ing.-iliac General†	38
21, 22	SC & IP	9	9	9SC 0IP	100 0. 0	3			
25	SC (tail)	8	8	3	38. 5	5			2
11, 13, 14, 17	IM	17	14	10	71. 4	5	2	Inguinal	2
12	SC & IM	2	2	2SC 0IM	100 0. 0	1			
Total		372	324	278	85. 8	152	20		42
Nitrogen mustard									
7, 8, 21, 23	SC	26	26	18	69. 2	16	—	—	9
Untreated									
11–16, 19	SC	47	39	19	48. 7	28	6	Inguinal	4
11, 12, 14, 17, 18	IM	20	19	14	73. 7	16	2	Inguinal	5
17	SC & IM	6	6	6SC 5IM	100 83. 3	5			3
Total		73	64	39	60. 9	49	8		12

*Abbreviations: SC = subcutaneous—flank unless otherwise designated; IP = intraperitoneal; IM = intramuscular—right thigh.
†Lymph glands, lungs.

Forty-seven puppies received subcutaneous flank inoculations only; 39 lived 7 days or longer and 19 (48.7%) of these developed growth recognizable by the 10th day. Subcutaneous transplants regressed completely in 4 dogs. Six of 28 animals living 16 days or longer had regional lymph-gland metastases.

Twenty puppies received only intramuscular inoculation of tumor cells; 19 lived for 7 days or longer; 14 (73.7%) developed tumors recognized by the 15th day. Regional lymph-gland metastases were noted in 2 of 16 animals living 16 days or longer.

The gross and histologic characteristics of the actively growing tumor in the nontreated animals were similar to those described in treated dogs. The regressing tumor had a central area of fibrous connective tissue interspersed among degenerating and necrotic tumor cells. Infiltrating cell types, such as lymphocytes and neutrophils, were frequently seen about the periphery of the necrotic center.

Although most of the puppies were injected either in the subcutis of the flank (usually bilaterally), or in the right gluteal-muscle mass, 3 small mixed-site groups may be noted in table 1 (*i.e.*, SC & IM, SC & IP). Transplant growth was noted in all dogs in these small groups, not only in the irradiated series, but also in the untreated one. It is felt that the number of instances here presented are numerically inadequate for proper evaluation, but that the phenomenon warrants future investigation.

Summary

A spontaneous canine thyroid carcinoma has been serially transplanted from the 7th through the 30th generation of mixed-breed canine hosts. Three hundred twenty-four puppies were X-irradiated at a level of 150 to 300 r, 85.8 percent of which developed transplant growth subsequent to inoculation with tumor cells in various sites. Twenty-six dogs were pretreated with nitrogen mustard, 69.2 percent of which developed transplant growth subsequent to inoculation of neoplastic cells in the subcutis of the flank. Sixty-four dogs were injected with neoplastic cells without any pretreatment; 60.9 percent of these developed transplant growths in various sites. In advanced generations, the tumor tissue was histologically similar to that of the first 6 serial passages. Metastases occurred occasionally in the X-irradiated and untreated groups, but not at all in the nitrogen mustard series. The regional lymph node was the most common site of involvement. Determination of complete regression could be made only in the subcutaneous area. Regressions occurred occasionally-to-frequently in all groups.

References

(*1*) Allam, M. W., Lombard, L. S., Stubbs, E. L., and Shirer, J. F.: Transplantation of a thyroid carcinoma within the canine species. Cancer Res. 15: 734–737, 1954.

(*2*) Craigie, J.: Survival and preservation of tumors in the frozen state. Advances Cancer Res. 2: 197–228, 1954.

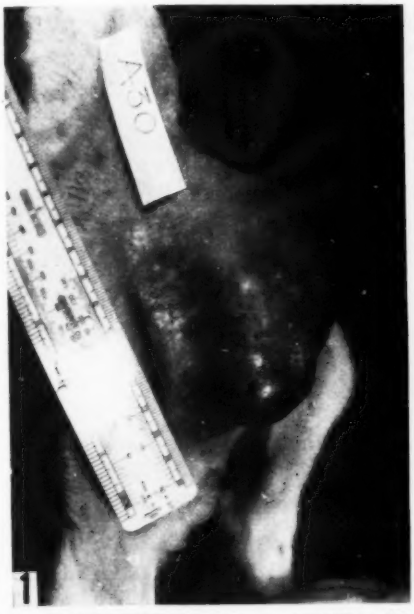
PLATE 17

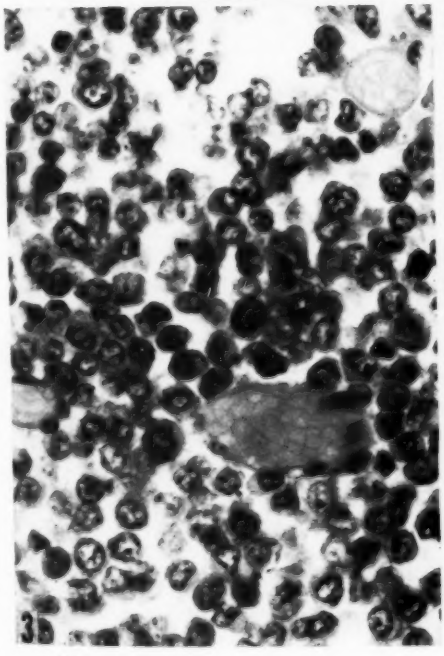
FIGURE 1.—The transplantable canine carcinoma 21 days following subcutaneous flank inoculation in a mixed-breed puppy from the 30th transplant series.

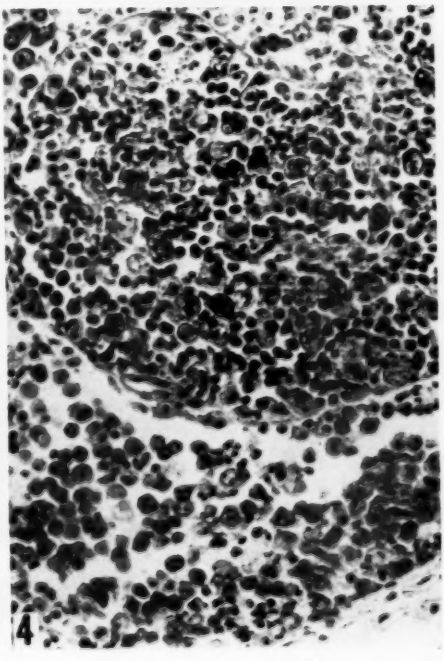
FIGURE 2.—Canine carcinoma metastases in iliac lymph glands of the animal pictured in figure 1, taken at necropsy on the 22nd day following transplantation.

FIGURE 3.—A biopsy of the canine carcinoma from the subcutaneous growth shown in figure 1, taken 14 days post inoculation. Several capillaries are prominent. × 600

FIGURE 4.—A section of the right iliac lymph gland seen in figure 2 illustrating canine carcinoma cells in the sinusoids. × 300







383538—56——6

Effect of Glutamine on the Growth and Metabolism of Liver Cells *in Vitro* [1]

B. B. Westfall, V. J. Evans, Ellison V. Peppers, Naomi M. Hawkins, *and* W. R. Earle, *Tissue Culture Section, Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

Ehrensvärd, Fischer, and Stjernholm (*1*) found that glutamine was a necessary growth factor for mammalian cells in tissue culture. It has become the custom to incorporate glutamine at the approximate level found in serum or plasma (*2*) into synthetic (*3–6*) or the known portion of semisynthetic (*7–9*) nutritional fluids for cells *in vitro*. Fischer (*8*) emphasized the key role of glutamine in the growth of cells in tissue culture. McIlwain found that certain streptococci needed glucose in the medium in order for the bacteria to use the glutamine (*10*). These observations, in part, prompted the present investigation of the effect of withholding glutamine on the growth and metabolism of 2 strains of cells originating from the parenchymal epithelial cells of the mouse liver. This investigation was of further interest in the light of the finding by Meister and Tice in 1950 (*11*) that glutamine could participate in transamination reactions with α-keto acids. The disappearance of glucose from the medium simultaneously with the production of lactic acid was studied in the presence and absence of glutamine. The changes in concentration of various amino acids and of pyruvic and α-ketoglutaric acids were followed. Particular attention was given to the change in concentration of the glutamine during the experimental period. It was found that in the absence of glutamine neither of the 2 cell strains could use glucose or maintain the inoculum; they were also unable to use appreciable amounts of the amino acids supplied to them, nor could they produce and return to the medium lactic, pyruvic, or α-ketoglutaric acids.

Experimental

The 2 cell strains from liver epithelium used for this study were: line 1469 derived from a single cell (*12*) of strain 721 (*13*), previously studied for its glycogen content (*14*), and the strain 1795 "high tumor" line derived in turn from line 1469. Line 1795 had undergone 1 animal passage through the mouse strain from which cell strain 721 was originally derived (*12,13,15*). Cultures of the cells were set up by the replicate-

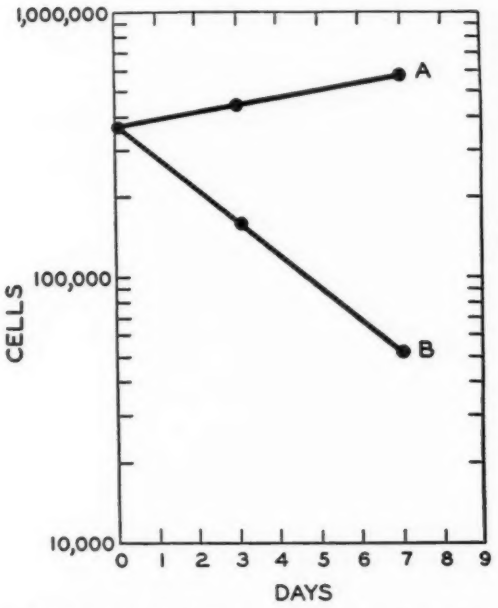
[1] Received for publication February 14, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

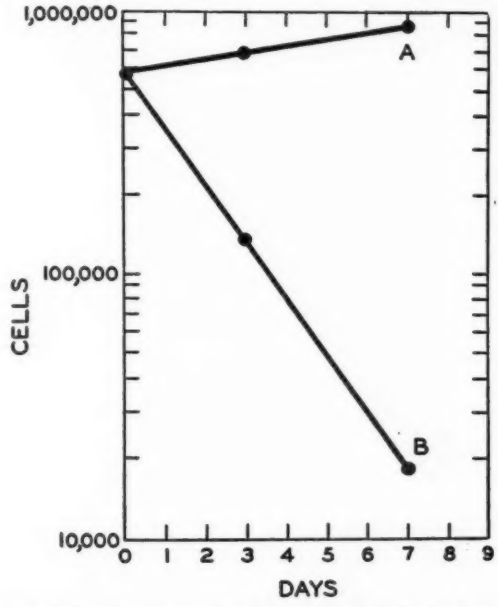
inoculum technique, using cell suspensions (*16*) and enumeration of nuclei (*17*). The inoculum aimed at was approximately half a million cells per T-15 flask. The medium contained the constituents of NCTC mixture 108 (*18*), from which the unsaturated fatty acids had been omitted, plus 3 mg. of protein nitrogen per 100 ml. as horse-serum residue (*19*). The free amino acids, amines, and amides of NCTC 108 supplied roughly twice this quantity of nitrogen. There were no detectable free amino acids supplied by the horse-serum solution. Exploratory analyses of the serum preparation indicated that as much as 8 percent of its nitrogen appeared as glutamic acid following acid hydrolysis. Presumably, in the protein the latter would be present as combined glutamine, *i.e.*, over 200 μg. N as glutamine combined in the serum would be present per 100 ml. of the medium. There is no evidence that the cells can use combined material of this sort. In order to study the effect of incubation alone on the medium, aliquots of the medium were handled in a fashion similar to the cell supernatant fluids, with incubation and mock fluid change, *i.e.*, the usual portion was withdrawn and replaced as for the cultures. Unincubated medium kept at 5° C. was analyzed for comparison. Glucose was estimated by an adaptation of the Sumner method without deproteinization of the medium (*20*). Lactic acid was estimated as previously described (*21*). α-Keto acids were estimated, after separation on paper, as the dinitrophenylhydrazones (*22*). The amino acids and glutamine were separated by paper chromatography (*22*). Glutamine and ammonia were also estimated by the Conway diffusion technique (*2,22,23*). In addition to estimation on paper, alanine was also estimated by conversion to the acetaldehyde (*24*). Each cell strain was cultured with glutamine (*A*) and without glutamine (*B*) in the culture fluid. The cells were carried for 7 days with 1 fluid change at the end of the 3rd day. The used medium was taken off at the end of the 3rd and 7th days following planting of each of the replicate cultures. Thus the fluid for analysis had been incubated with the cells for periods of 3 and 4 days. The acellular medium had likewise been incubated for these intervals. Analyses were made on pooled fluid from a minimum of 3 flasks. Protein-free ultrafiltrates of the fluids were used for the studies of the nonprotein nitrogen content (*2*).

Results

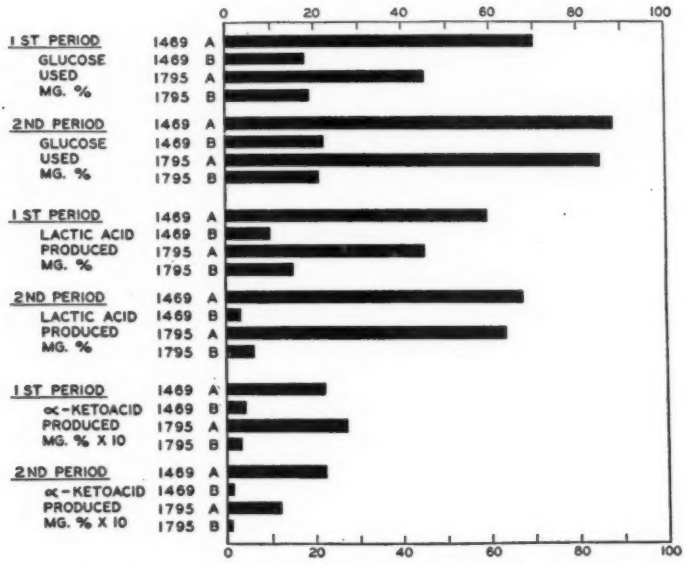
The cell content of the initial inoculum per flask and the final numbers are shown in text-figure 1 for strain 1469 and in text-figure 2 for strain 1795. It is apparent that in both strains the cells lacking only glutamine (*B*) were dying rapidly compared with the controls (*A*). With glutamine present the increase in population numbers was approximately 50 percent in 7 days. Text-figure 3 shows the gross total disappearance of glucose, in mg. per 100 ml., from the medium and the excess lactic acid produced in the medium during the intervals. Total production of α-keto acids is also shown. Pyruvic acid represents about 60 percent of the values shown, and α-ketoglutaric acid about 20 percent—the rest being made up



TEXT-FIGURE 1.—Cell inoculum and final population numbers for strain 1469. (*A*) with and (*B*) without added glutamine.



TEXT-FIGURE 2.—Initial cell inoculum and final population numbers for strain 1795. (*A*) with and (*B*) without added glutamine.



TEXT-FIGURE 3.—Glucose, lactic acid, and α-keto acids in the used medium at the 3-day (1st period) and 4-day (2nd period) fluid changes. The figures represent glucose disappearance and amount of lactic and α-keto acids produced. Glucose in the starting medium was 100 mg. per 100 ml.; there were no lactic, pyruvic, or α-ketoglutaric acids in the original medium.

of unknown substances separable by paper chromatography from these two. The changes in concentration of arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, found by Fischer (*8*) to be essential for the growth of cells in tissue culture, are shown in table 1. In addition tyrosine, found by Eagle (*9*) to be necessary for growth of strain L and HeLa cells,

TABLE 1.—*Percentage change in concentration of amino acids in used medium from 4-day period for strain 1469, series* (A)*

Alanine	+25
Arginine	−25
Cysteine	−10
Glutamic acid	+10
Glutamine	−30
Histidine	−50
Isoleucine	−50
Leucine	−25
Lysine	−10
Methionine	−50
Phenylalanine	−50
Threonine	−25
Tryptophan	−25
Tyrosine	−25
Valine	−25

*Within the limits of the methods the results for strain 1795 for the same interval were similar. There were no detectable changes in the amino acid content of either (*B*) series. There was no urea in the fresh medium and none could be detected after growth of the cells. All medium (*A*) samples showed some apparent ammonia, evidently derived mostly from the glutamine by slow deamidation.

is included. Since alanine and glutamic acid had increased in concentration these are likewise included. Data are detailed only for strain 1469, series (*A*), since the results for 1795 (*A*) were very similar. There were no detectable changes in the nitrogen distribution of the (*B*) series. There should, perhaps, have been some slight increase in free amino acids contributed by the death and disruption of the cells in the glutamine-free medium. If such increases occurred they were below detection in the background of amino acids furnished by the medium.

Discussion

In the 7-day interval the cells of strain 1469 in a single flask grew from 370,000 to 580,000, an increase in cell numbers of 210,000. Liver epithelial cells have been found to weigh (*14*) approximately 3 mg. per million cells so that the above increment represents a 630 γ increase. Similar cells in culture have been found to have approximately 18 percent protein, so that the increment would represent 114 γ protein or about 19 γ protein nitrogen. The data in text-figure 3 indicate that for this amount of protein nitrogen to be formed and retained, 4740 γ of glucose were consumed in the process of maintaining the cells and forming the new cells and their protein. On a molar basis it would appear that roughly 20 moles of glucose disappear per mole of nitrogen incorporated into cellular protein. The data also indicate that for the (*A*) categories the lactic acid remaining in the supernatant does not account for all the glucose disappearing from the supernatant fluid. When the 2 cell strains are compared for glucose utilization, lactic acid production, and α-keto acid production (as in table 2), it is apparent that strain 1469 was utilizing glucose at from 1½ to 2 times the rate for strain 1795. Lactic acid-production ratios paralleled the glucose utilization ratios. To make conditions as nearly comparable as possible the results have been adjusted to the 100,000-cell basis for each strain. These calculations have been made only for the (*A*) categories, since it was considered invalid to make such calculations for rapidly dying populations of the (*B*) group where the only figures available would be those for the terminal survivals. Sufficient information is not available to permit one to state firmly that differences in population density influence the rate of glucose disappearance.

The incubated medium of the (*B*) series yielded 50 γ N as NH_3 in the Butler procedure and 180 γ N as apparent amide N per 100 ml., all derived

TABLE 2.—*Glucose removed and lactic and α-keto acids produced in medium during growth of cells of* (A) *series. Mg. per 100,000 cells in culture*

Substance	First period (*3 days*)		Ratio 1469/1795	Second period (*4 days*)		Ratio 1469/1795
	1469 (*A*)	1795 (*A*)		1469 (*A*)	1795 (*A*)	
Glucose.....	17. 3	7. 5	2. 3	17. 6	11. 0	1. 5
Lactic acid....	14. 5	7. 4	2. 0	13. 6	8. 2	1. 7
α-Keto acids..	0. 52	0. 44	1. 2	0. 44	0. 15	2. 9

from the various compounds, such as cobalamin, nicotinamide, thiamin, and pyridoxamine, in the chemically known portion of the medium as shown by control experiments. The incubated media for the series 1469 and 1795 gave an additional 170 γ NH_3 in the 4-day incubation, indicating slight deamidation of the glutamine and asparagine at *p*H 7.4 and 37.5° C. in the absence of cells. There was no detectable increase in glutamic acid for the media during this interval when examined by paper chromatography. It appears very probable that this small decomposition of the glutamine is the spontaneous sort noted by Bray *et al.* (*25*) in phosphate-containing buffer at 37° C. The end products are pyrrolidonecarboxylic acid and ammonia. The rate of decomposition noted in the present experiments was much less than in the experiments of earlier workers using simple phosphate buffer as the medium. Beyond the spontaneous breakdown of the glutamine in the medium the deamidation of this amide owing to the presence of the cells was small, representing only 140 γ amide N per 100 ml. used medium in the second or 4-day interval for either of the (*A*) series. This amount was 4.7 γ per T-15 flask. Owing to the fact that this figure is derived as the difference of 3 separate estimations, the factor of uncertainty is large. The estimation on paper indicated that one third more than this quantity had been converted to non-amide N. The used media in the (*B*) series showed a small amount of "glutamine-reacting" material, 180 γ per 100 ml. on the 7th day, evidently originating from the decomposition of the cells in a medium where the cells were rapidly dying.

The increase in alanine shown by both (*A*) groups is interesting since it suggests transamination and apparently is dependent under these circumstances on the presence of the cells, although it has been shown that given proper precursors, horse-serum residue contains a sufficient concentration of transaminating enzyme to convert pyruvic acid to alanine. However, with the horse-serum residue representing only 3 mg. N per 100 ml. of the medium and no added pyruvic acid initially present, it was not possible to demonstrate any increase in alanine on incubation of the medium alone. The total amino acids disappearing from the medium seem to be considerably more than can be accounted for as protein N in the new cellular mass formed in the (*A*) series. This suggests that pathways other than fixation as cellular protein nitrogen alone may operate. Since this work was completed, Pasieka, Morton, and Morgan (*26*) have published work with explants of embryonic chick heart in which they have found an increase of both glutamic acid and alanine during growth of the cells. The ability to increase the concentration of these constituents of the medium is, therefore, not peculiar to the liver cells.

Summary

The 2 mouse liver cell strains 1469 and 1795 derived from strain 721, of parenchymal epithelial origin, have been cultivated in media with and without added glutamine. Horse-serum protein contributed 3 mg. N per 100 ml. of the medium. With glucose present both cell strains grew slowly in the presence of glutamine, and both died in its absence; per tissue

unit, strain 1469 used glucose and produced lactic acid at about twice the rate of strain 1795. In the absence of added glutamine the cells of both strains were not able to use glucose or the amino acids nor could they produce lactic or α-keto acids.

References

(*1*) Ehrensvärd, G., Fischer, A., and Stjernholm, R.: Protein metabolism of tissue cells in vitro. 7. The chemical nature of some obligate factors of tissue cell nutrition. Acta physiol. scandinav. 18: 218–230, 1949.

(*2*) Westfall, B. B., Peppers, E. V., Sanford, K. K., and Earle, W. R.: The amino acid content of the ultrafiltrate from horse serum. J. Nat. Cancer Inst. 15: 27–35, 1954.

(*3*) Morgan, J. F., Morton, H. J., and Parker, R. C.: Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exper. Biol. & Med. 73: 1–8, 1950.

(*4*) Healy, G. M., Fisher, D. C., and Parker, R. C.: Nutrition of animal cells in tissue culture. X. Synthetic medium No. 858. Proc. Soc. Exper. Biol. & Med. 89: 71–77, 1955.

(*5*) Morgan, J. F., and Morton, H. J.: Studies on the sulfur metabolism of tissues cultivated *in vitro*. I. A critical requirement for L-cystine. J. Biol. Chem. 215: 539–545, 1955.

(*6*) Evans, V. J., Bryant, J. C., Fioramonti, M. C., McQuilkin, W. T., Sanford, K. K., and Earle, W. R.: Studies of nutrient media for tissue cells *in vitro*. I. A protein-free chemically defined medium for cultivation of strain L cells. Cancer Res. 16: 77–86, 1956.

(*7*) White, P. R., and Waymouth, C.: Chemically defined synthetic nutrients for tissue cultures for pharmacological studies. Ann. New York Acad. Sc. 58: 1023–1028, 1954.

(*8*) Fischer, A.: On the protein metabolism of tissue cells *in vitro*. J. Nat. Cancer Inst. 13: 1399–1423, 1953.

(*9*) Eagle, H.: Nutrition needs of mammalian cells in tissue culture. Science 122: 501–504, 1955.

(*10*) McIlwain, H.: Metabolism and functioning of vitamin-like compounds. I. Ammonia formation from glutamine by haemolytic streptococci: its reciprocal connexion with glycolysis. Biochem. J.: 40: 67–78, 1946.

(*11*) Meister, A., and Tice, S. V.: Transamination from glutamine to α-keto acids. J. Biol. Chem. 187: 173–187, 1950.

(*12*) Hobbs, G. L., Sanford, K. K., Evans, V. J., and Earle, W. R.: Establishment of a clone of mouse liver cells from a single cell. In preparation.

(*13*) Evans, V. J., Earle, W. R., Wilson, E. P., Waltz, H. K., and Mackey, C. J.: The growth *in vitro* of massive cultures of liver cells. J. Nat. Cancer Inst. 12: 1245–1265, 1952.

(*14*) Westfall, B. B., Evans, V. J., Shannon, J. E., Jr., and Earle, W. R.: The glycogen content of cell suspensions prepared from massive tissue culture: comparison of cells derived from mouse connective tissue and mouse liver. J. Nat. Cancer Inst. 14: 655–664, 1953.

(*15*) Hawkins, N. M., Evans, V. J., and Earle, W. R.: Studies on hepatoma lines derived from mouse liver epithelial cells grown for long term in tissue culture. In preparation.

(*16*) Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E., and Waltz, H. K.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907–927, 1951.

(*17*) Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(*18*) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. II. An improved, protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. 16: 87–94, 1956.

(*19*) SANFORD, K. K., WALTZ, H. K., SHANNON, J. E., JR., EARLE, W. R., and EVANS, V. J.: The effects of ultrafiltrates and residues of horse serum and chick-embryo extract on proliferation of cells *in vitro*. J. Nat. Cancer Inst. 13: 121–137, 1952.

(*20*) HENDRIX, J. P., WESTFALL, B. B., and RICHARDS, A. N.: Quantitative studies of the composition of glomerular urine. XIV. The glomerular excretion of inulin in frogs and necturi. J. Biol. Chem. 116: 735–747, 1936.

(*21*) WESTFALL, B. B., PEPPERS, E. V., and EARLE, W. R.: The change in concentration of certain constituents of the medium during growth of the strain HeLa cells. Am. J. Hyg. 61: 326–333, 1955.

(*22*) ———.: α-Keto-acids in tissue culture. I. The concentration of pyruvic and α-ketoglutaric acids in the ultrafiltrates from horse serum and chick-embryo extract. J. Nat. Cancer Inst. 16: 337–347, 1955.

(*23*) BUTLER, G. W.: Determination of glutamine and asparagine in plant tissue extracts. Anal. Chem. 23: 1300–1304, 1951.

(*24*) ALEXANDER, B., and SELIGMAN, A. M.: A colorimetric method for the micro-determination of α-alanine in blood. J. Biol. Chem. 159: 9–19, 1945.

(*25*) BRAY, H. G., JAMES, S. P., RAFFAN, I. M., and THORPE, W. V.: The enzymic hydrolysis of glutamine and its spontaneous decomposition in buffer solutions. Biochem. J. 44: 625–627, 1949.

(*26*) PASIEKA, A. E., MORTON, H. J., and MORGAN, J. F.: The metabolism of animal tissues cultivated *in vitro*. I. Amino acid metabolism of chick embryonic-heart fibroblasts cultivated in synthetic medium M 150. J. Nat. Cancer Inst. 16: 995–1009, 1956.

Daily Eosinophil Rhythm in Mice Bearing a Transplanted Mammary Carcinoma [1, 2]

FRANZ HALBERG, HALVOR VERMUND, *and* JOHN J. BITTNER, *Division of Cancer Biology of the Department of Physiology and Section of Radiation Therapy of the Department of Radiology, University of Minnesota Medical School, Minneapolis, Minnesota*

A decrease in the number of circulating eosinophil cells occurs in the morning in diurnally active human beings, in conjunction with other physiologic changes noted in indices of adrenal function (for review *see 1*). In the nocturnal mouse (*2–4*) or rat (*5*), physiologic eosinopenia occurs at night. The daily periodic changes in number of eosinophils are inhibited by adrenalectomy in mice (*6,7*) and in man (*8,9*) and are reinduced—in the absence of the adrenals—by the periodic administration of adrenal corticosteroids (*1,9*). The adrenal glands may therefore be regarded as part of an intrinsic mechanism of daily periodicity. But it has also been shown that extrinsic factors act as the synchronizers of the intrinsic mechanism (*1,10*). Thus, in mice, the lighting regimen will be primarily responsible for the temporal placement within the 24-hour period of the daily eosinophil rhythm (*6,10*). By standardizing those factors which affect the eosinophil rhythm from without (like lighting), eosinophil counts may be used for the study of factors from within which maintain the rhythm—that is, for the study of adrenal function. The present study was carried out under standardized environmental circumstances in order to explore eosinophil behavior—and thus one aspect of cyclic adrenal function—in the presence of a transplanted rapidly growing adenocarcinoma of the breast.

Materials and Methods

Two experiments were carried out on $AxZbF_1$ and $ZbAxF_1$ male mice. ZBC female mice, obtained by backcrossing $AxZbF_1$ or $ZbAxF_1$ hybrids to the Z (C3H) parents were used for a third experiment. The mice (about 2 months of age at the time of transplantation) were housed individually in a room maintained at about 78° F. which was illuminated during the day and kept dark at night. Purina Fox Chow Checkers and tap water were available to the mice *ad libitum* after weaning and throughout the study.

Two spontaneous mammary carcinomas were the source of the tumor

[1] Received for publication March 5, 1956.

[2] This investigation was supported in part by the Elsa U. Pardee Foundation and by research grants from the American Cancer Society, the U. S. Public Health Service (G-4158), and the Graduate School of the University of Minnesota.

transplants. One had developed in an AZF_1 female about 2 years prior to this study and was used experimentally after a number of passages through AZF_1 or $AxZbF_1$ male mice. The other had developed in a Z female about 1 year previously and was maintained by repeated serial passage in ZBC male mice. Both these tumors had been identified by microscopic examination as adenocarcinomas.

For each experiment, a single tumor was dissected out and passed through a tissue press with holes 1 mm. in diameter, following which the tissue pulp was suspended in sterile 0.9 percent saline solution. A volume of 0.1 cc. of a 20 percent suspension was inoculated subcutaneously into the lower back of each mouse of one group, while a comparable group of mice was injected with the same volume of sterile 0.9 percent saline solution.

Eosinophil counts were obtained by assembly-line procedures previously described (*11*) during 2 distinct time periods, namely, from 7 to 11 A. M. (day) and from 9 to 12 P. M. (night). At each time of day, tumor-inoculated and saline-injected mice were sampled in rotation in order to reduce the possible effect of a difference between the 2 groups due to a time factor. Furthermore, separate subgroups of the tumor-inoculated and the saline-injected groups were used for eosinophil counts during the day and at night. "Within-day" sampling was thus serially independent in all the experiments.

In the first experiment, counts were made only at 15 days after inoculation. During the course of the second and the third experiments, the respective groups of mice were restudied at intervals of several days (*see* Results). Sampling at "day intervals" was thus serially dependent. Admittedly, the slight but significant effect of repeated venesections, which has been discussed elsewhere (*12*), was not controlled in these experiments.

More than 90 percent of the animals had developed palpable tumors 7 days after inoculation, and the incidence of tumor development approached 100 percent on the 15th day after inoculation. At that time, most of the tumors were large and many had started to ulcerate.

Results

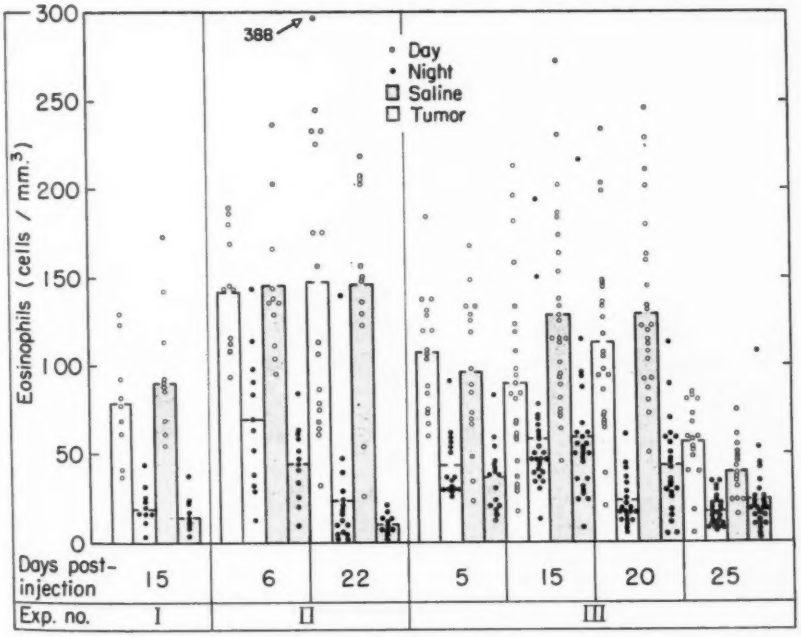
The variability in eosinophil level encountered in this investigation within the various subgroups of mice is shown in text-figure 1, but the analysis of the sources of variation is beyond the scope of this report.[3] Despite this variability within the subgroups, the differences in mean counts between day and night stand out clearly in text-figure 1 for both groups investigated and at all intervals after the injection. It seems fair to suggest, from this figure and from the summary of means and standard errors presented in table 1, that the 24-hour eosinophil rhythm constitutes a characteristic feature of the populations of mice studied, with and without a transplanted rapidly growing mammary carcinoma. This suggestion is further supported by the results of a statistical analysis of the data employing Student's t test (table 1). At each time point studied, the day-

[3] Those factors which may contribute to the variability in number of eosinophils have been discussed (*1*).

night difference in mean eosinophil count is significant below the 1 percent level for the tumor-inoculated as well as for the saline-injected mice.

A reduction in count observed in the third experiment at the 25th day after tumor inoculation remains unexplained (*see* text-figure 1). But it is pertinent to note that in this experiment a similar reduction in count occurred in the saline-injected group at the same time interval after injection. Hence, this reduction in eosinophil count cannot be interpreted as dependent upon the presence of a growing tumor. Furthermore, a reduction in the eosinophil count could not be detected at the 22nd day after injection in the second experiment.

It may also be recalled that on any one day of experiment and for either of the 2 groups studied, the subgroups of mice sampled during the day and at night were not the same. Superficially, this may seem to constitute a reservation as to the validity of the percentage changes shown in the last column of table 1. In this column, the magnitude of day-night change in mean count is computed as the change from the mean day count to the mean night count, with the mean day count equated to 100 percent. The changes thus computed are considerable in extent and consistent in direction for the tumor-inoculated as well as for the saline-injected groups. If, as seems likely, the subgroups studied during the day and at night were comparable, the provision for serially independent sampling may constitute an advantageous aspect of experimental design; all the data are obtained from mice used for venesection only once during a given 24-hour



TEXT-FIGURE 1.—Day-night differences in eosinophil count of mice with and without a transplanted adenocarcinoma at several time points after inoculation of tumor.

TABLE 1.—*Eosinophil rhythm in mice with and without transplanted adenocarcinoma**

Expt. No.	Stock, sex, and tumor	Days post inject.	R_x	Time	No. of mice	Eosinophils mean ± S.E.	Difference ± S.E.†	Percent change
I	AxZbF$_1$♂ T31	15	T	D	9	79±11	58±11	−74
				N	10	20± 4		
			S	D	10	97±12		
				N	9	16± 3	81±13	−83
II	AxZbF$_1$♂ and ZbAxF$_1$♂ T32	6	T	D	11	143±10	75±15	−52
				N	12	69±11		
			S	D	11	145±13		
				N	12	46± 6	100±14	−69
		22	T	D	17	148±23	124±25	−84
				N	16	23± 8		
			S	D	12	147±18		
				N	11	10± 1	137±18	−93
III	ZBC♀ K32	5	T	D	16	107± 8	66± 9	−61
				N	16	42± 5		
			S	D	16	96±11		
				N	16	36± 5	60±12	−62
		15	T	D	25	90±11	33±13	−36
				N	26	57± 7		
			S	D	26	128±11		
				N	26	59± 8	69±13	−54
		20	T	D	23	112±11	90±12	−80
				N	22	23± 3		
			S	D	23	134±51		
				N	23	42± 6	92±12	−69
		25	T	D	16	56± 6	39± 5	−71
				N	26	16± 1		
			S	D	15	40± 4		
				N	26	24± 4	16± 6	−41

*T=Breast-tumor suspension in 0.9 percent saline solution; S=0.9 percent saline solution; D=from 7 to 10 A. M.; N=from 9 to 12 P. M.

†Mean eosinophil count obtained for day sample *minus* the corresponding night value; numbers are rounded; all are significant below the 1 percent level.

period. At least the "within-day" effect upon eosinophil count of a previous venesection is thereby eliminated. But irrespective of the considerations noted above, the main point of this report, namely, the occurrence of day-night changes in eosinophil level of mice bearing a transplanted mammary carcinoma, appears to be established.

Discussion

Among the various phenomena encountered in body functions in a 24-hour period, the daily rhythm in cell division has been of obvious interest to students of normal and malignant growth. The status of our knowledge on mechanisms of mitotic periodicity has recently been discussed (*1,5,13*). Furthermore, with one exception (*14*), the available data on mitotic behavior in cancers have been interpreted as indicative of the obliteration of the 24-hour mitotic rhythm (*15,16*). But largely unexplored is the behavior, in the presence of cancer, of other 24-hour-period phenomena which may be related to the mitotic periodicity (*4*). Since adrenal corticosteroids depress certain mitoses (*17–21*), as well as eosinophils, and since both these effects involve 24-hour periodicity, the behavior of the daily eosinophil rhythm in cancerous mice appeared to be of interest. The qualitative question raised herein as to the presence or the absence of eosinophil rhythm in mice bearing a certain transplanted tumor may be answered in the affirmative. Even though data were obtained at only 2 times during the day, they seem sufficient to suggest that the day-night differences in the number of circulating eosinophils of the tail blood of the mouse are not abolished by the transplantation of a fast-growing mammary carcinoma. This suggestion may apply to several stages of tumor growth. By 15 days after inoculation, over 50 percent of the tumors were either in their exponential phase of growth or had already started to ulcerate. The data from 5 to 25 days following the injection thus describe various stages of tumor development, all of which are associated with statistically significant day-night differences in eosinophil count. The eosinophil rhythm and, thus, the periodic function of the adrenal mechanisms controlling it (*1*) seem to characterize the physiology of the host bearing at least 1 type of transplanted tumor. The exploration of more subtle effects of the presence of a transplanted tumor upon the period, amplitude, or phase of eosinophil rhythm must await the analysis of data obtained with more frequent sampling. Likewise, eosinophil behavior of mice with spontaneous tumors remains to be investigated. In this connection, it seems of interest that for Z females bearing a spontaneous breast tumor, an inhibition of day-night difference in mitoses of pinna epidermis has recently been noted in this laboratory.

Summary

Mice of the $ZbAxF_1$, $AxZbF_1$, and ZBC stocks exhibit significant day-night differences in counts of circulating tail-blood eosinophils. Significant day-night differences in eosinophil counts also are exhibited by such mice following the transplantation and growth of mammary carcinoma.

References

(*1*) HALBERG, F.: Some physiological and clinical aspects of 24-hour periodicity. Journal-Lancet 73: 20–32, 1953.

(*2*) HALBERG, F., and VISSCHER, M. B.: Regular diurnal physiological variation in eosinophil levels in five stocks of mice. Proc. Soc. Exper. Biol. & Med. 75: 846–847, 1950.

(*3*) PANZENHAGEN, H., and SPEIRS, R.: Effect of horse serum, adrenal hormones, and histamine on the number of eosinophils in the blood and peritoneal fluid of mice. Blood 8: 536–544, 1953.

(*4*) LOUCH, C., MEYER, R. K., and EMLEN, J. T.: Effect of stress on diurnal fluctuations in eosinophils of the laboratory mouse. Proc. Soc. Exper. Biol. & Med. 82: 668–671, 1953.

(*5*) HALBERG, F., ZANDER, H. A., HOUGLUM, M. W., and MÜHLEMANN, H. R.: Daily variations in tissue mitoses, blood eosinophils and rectal temperatures of rats. Am. J. Physiol. 177: 361–366, 1954.

(*6*) HALBERG, F., VISSCHER, M. B., and BITTNER, J. J.: Observations on the eosinophil rhythm in mice; range of occurrence, effects of illumination, feeding, and adrenalectomy. Am. J. Physiol. 174: 109–122, 1953.

(*7*) BROWN, H. E., and DOUGHERTY, T. F.: The diurnal variation of blood leucocytes in normal and adrenalectomized mice. Endocrinology 58: 365–375, 1956.

(*8*) HALBERG, F., VISSCHER, M. B., FLINK, E. B., BERGE, K., and BOCK, F.: Diurnal rhythmic changes in blood eosinophil levels in health and in certain diseases. Journal-Lancet 71: 312–319, 1951.

(*9*) KAINE, H. D., SELTZER, H. S., and CONN, J. W.: Mechanism of diurnal eosinophil rhythm in man. J. Lab. & Clin. Med. 45: 247–252, 1955.

(*10*) HALBERG, F., VISSCHER, M. B., and BITTNER, J. J.: Relation of visual factors to eosinophil rhythm in mice. Am. J. Physiol. 179: 229–235, 1954.

(*11*) HALBERG, F., BITTNER, J. J., and VISSCHER, M. B.: Tail blood eosinophil levels in several inbred strains of mice under standard conditions. Blood 6: 832–837, 1951.

(*12*) HALBERG, F.: Changes in eosinophil count of mice with venisections repeated at intervals of several days. Proc. Soc. Exper. Biol. & Med. 82: 160–162, 1953.

(*13*) BULLOUGH, W. S.: The energy relations of mitotic activity. Cambridge Biol. Rev. 27: 133, 1952.

(*14*) VOUTILAINEN, A.: Über die 24-Stunden-Rhythmik der Mitosenfrequenz in malignen Tumoren. Acta path. et microbiol. scandinav. Suppl. IXC. 1–104, 1953.

(*15*) DUBLIN, W. B., GREGG, R. O., and BRODERS, A. C.: Mitosis in specimens removed during day and night from carcinoma of large intestine. Arch. Path. 30. 893–895, 1940.

(*16*) BLUMENFELD, C. M.: Studies of normal and of abnormal mitotic activity II. The rate and the periodicity of the mitotic activity of experimental epidermoid carcinoma in mice. Arch. Path. 35: 667–673, 1943.

(*17*) GREEN, H. N., and GHADIALLY, F. N.: Relation of shock, carbohydrate utilization, and cortisone to mitotic activity in the epidermis of the adult male mouse. Brit. M. J. 1: 496–498, 1951.

(*18*) STUDER, A., and FREY, J. R.: Wirkung von Cortison auf die ruhende und die mit Vitamin A oder Testosteronpropionat zur Proliferation gebrachte Epidermis der Ratte. Dermatologica 104: 1–18, 1952.

(*19*) BULLOUGH, W. S.: Stress and epidermal mitotic activity. I. The effects of the adrenal hormones. J. Endocrinol. 8: 265–274, 1952.

(*20*) TEIR, H., and ISOTALO, A.: Influence of cortisone on mitosis. I. Effect of single dose and prolonged application. Ann. med. exper. et biol. Fenniae 31: 171–180, 1953.

(*21*) CHAUDHRY, A. P., HALBERG, F., and BITTNER, J. J.: Reduction of mitotic activity in pinna epidermis of mice given cortisol or 9 α-fluorocortisol. Proc. Soc. Exper. Biol. & Med. 91:602–604, 1956.

Enzyme Changes Induced in Normal and Malignant Tissues with Chemical Agents. VII. Effect on Hydrolytic and Synthetic Enzymes of Diphosphopyridine Nucleotide in Sarcoma 37 [1]

V. S. WARAVDEKAR,[2] O. H. POWERS, *and* J. LEITER,
Laboratory of Chemical Pharmacology, National Cancer Institute,[3] Bethesda, Maryland

It was reported in a previous communication (*1*) that the tumors from animals treated with α-peltatin showed approximately 40 percent decreased ability to utilize glucose than the tumors from the untreated mice. In part, this might have been due to diminished activity of such enzymes as adenosinetriphosphatase, hexokinase, or aldolase. It was also considered possible that a marked loss in endogenous diphosphopyridine nucleotide (DPN) in tumors following the administration of drug (*1*) might contribute to the low glucose utilization by the damaged tumors.

Diphosphopyridine nucleotide generally has been considered to be associated with particulate enzymes, and it is conceivable that in damaging tumor cells by the action of the drug the endogenous DPN may be exposed to the action of the enzymes that possess the ability to degrade DPN. It is known that there are 2 positions in the DPN molecule where degradation of DPN occurs: first, the cleavage of the glycosidic bond between nicotinamide and the rest of the molecule catalyzed by diphosphopyridine nucleotidase (DPNase) (*2*) and second, the cleavage of the pyrophosphate bond between adenylic acid and nicotinamide mono-nucleotide (NMN) catalyzed by diphosphopyridine nucleotide pyrophosphatase (DPN pyrophosphatase) (*3*). On the other hand, it is also likely that the tumor-damaging compound may interfere with the process of DPN synthesis in tumor cells, resulting in the lowered level of DPN in such tissues. Either explanation, if substantiated, could form a basis for the decreased ability of the treated tumor tissue to utilize glucose.

The present paper, therefore, deals with the possible effect of a tumor-damaging agent in relation to DPNase and DPN-pyrophosphatase activities, as well as the formation of DPN from NMN by tumor tissues.

Methods and Materials

Mice of strain CAF_1 [(BALB/c × A)F_1] bearing 6-day-old intramuscular implants of sarcoma 37 were employed for the most part. Other strains

[1] Received for publication March 23, 1956.
[2] Visiting Scientist, National Institutes of Health, Bethesda, Md.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

of mice bearing different tumors were used as indicated in the text. The tumor-damaging compound *acetylpodophyllotoxin-ω-pyridinium chloride* (NCI-3022) (*4*) was injected subcutaneously into mice bearing sarcoma 37 and the animals were sacrificed in groups of 4 at intervals of 2 to 24 hours after injection. Homogenates of normal and tumor tissues were prepared as described previously (*5*). Other tumors employed in this study were those described in our previous communication (*6*).

DPNase activity of the tissue homogenate was determined essentially according to the method of Colowick, Kaplan *et al.* (*7,8*). Potassium phosphate buffer, 0.3 ml., 0.1 *M*, *p*H 6.8, was added to 3 tubes. The first and the third tube contained 0.1 ml. of 2.3 m*M* DPN (Sigma "90"), the second and the third contained 0.1 ml. of enzyme in appropriate dilution, with distilled H_2O in the first 2 to make 0.5 ml. The tubes were incubated at 37.5° C. for 8 minutes. KCN, 2.5 ml., 1.2 *M*, *p*H 11.0, was added to these tubes; they were read at 340 mμ against a blank containing buffer and KCN. Subtracting the optical density obtained with the second tube from that of the third and subtracting this value in turn from the optical density of the first gave the amount of DPN split by the enzyme. The amount of DPN was calculated by the use of 5.28×10^6 cm.^{2} per mole as the molecular extinction coefficient for the DPN-cyanide complex at 340 mμ. This value is slightly higher than that (5.15) reported by Colowick *et al.* (*7*). Diphosphopyridine nucleotide-pyrophosphatase activity was determined in a similar way with the use of 0.3 ml. of 0.1 *M* potassium phosphate buffer, *p*H 6.8, and other constituents as described before, except that 0.1 ml. of 2 *M* nicotinamide (*2,3,9*) was added to the tubes before the enzyme to inhibit the DPNase activity present in the tissues. The reaction was stopped by heating the tubes at 100° C. in boiling water for 2 minutes. They were cooled and 2.2 ml. of 0.5 *M* ethanol in 0.1 *M* tris (hydroxymethyl)aminomethane buffer, *p*H 10.1, were added, followed by the addition of 0.2 ml. of alcohol dehydrogenase (300 μg. protein per ml.) to convert DPN completely into the reduced form (*10*). Conversion occurred in several minutes. The optical density at 340 mμ was then recorded. The amount of DPN split by the enzyme was calculated as before, using 6.27×10^6 cm.^{2} per mole as the molecular extinction coefficient for reduced DPN (*11*).

The combined activity of DPNase and DPN pyrophosphatase in tissue homogenates for DPN degradation was estimated by employing the procedure followed for the determination of DPN-pyrophosphatase activity of the homogenates except that nicotinamide was omitted in the reaction mixture for these determinations. Measurements of the total activity for DPN hydrolysis of most of the tissue homogenates were in close agreement with the sum of the DPNase and DPN-pyrophosphatase activity of these homogenates measured separately.

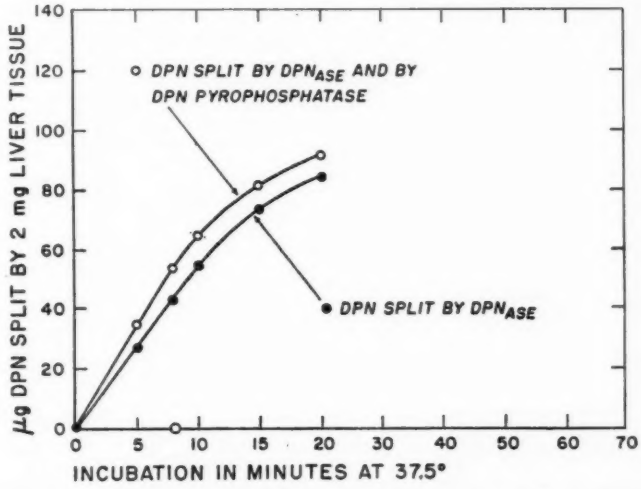
The rate of synthesis of DPN[4] by the tumor homogenate from drug-treated animals was followed according to the procedure of Kornberg (*12*)

[4] Nicotinamide mono-nucleotide was kindly furnished by Dr. N. O. Kaplan, The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md.

and of Hogeboom and Schneider (*13*). The reaction proceeded linearly at 10 and 20 minutes and was directly proportional to the amount of enzyme added. From this the micromoles of DPN formed per hour per milligram of tissue nitrogen was calculated. The nitrogen content of the tissue homogenates was determined by the micro-Kjeldahl method of Ma and Zuazaga (*14*).

Results

Degradation of DPN by liver homogenate at different intervals of incubation.—In order to determine the capacity of tissue to degrade DPN, an experiment was carried out by incubating liver homogenate with DPN. Text-figure 1 shows the effect of DPN-splitting enzymes of liver on DPN during incubation at several intervals. The rate of reaction to degrade DPN was linear for about 10 minutes of incubation of tissue with DPN.



TEXT-FIGURE 1.—Degradation of DPN by liver homogenate at different intervals of incubation.

DPN-splitting activity of various transplanted tumors.—Results on the degradation of DPN by some of the transplanted tumors commonly used in this laboratory are given in table 1. DPNase, DPN pyrophosphatase, and the combined activity of the 2 enzymes have been expressed in terms of micrograms of DPN split by 0.1 mg. of tissue nitrogen in 8 minutes at 37.5° C. In most of the tissues the sum of DPNase and DPN-pyrophosphatase activities nearly equaled the combined activity of these enzymes present in the tissue. In all the tissues, whether neoplastic or normal, the DPNase activity was much higher than the DPN-pyrophosphatase activity. The DPNase activity of the liver was about 1½ to 2 times greater than that of the tumors. The DPNase activity of the tumors varied from 28 to 48; the lowest activity was shown by the Krebs-2

ascites tumor and lymphoma #1 while the highest level of DPNase activity was present in sarcoma 37. The pyrophosphatase activity of these tumors ranged from 8 to 21. Of the other normal tissues, spleen had about 1½ times the activity of liver or kidney. DPNase activity of the spleen from animals bearing leukemia L1210 was about one half the activity of the normal spleen.

TABLE 1.—*Degradation of DPN by DPNase and DPN pyrophosphatase from homogenates of tumor and of organs*

Tissues*	Days after tumor implantation	Enzyme capacity to degrade DPN†		
		Combined	DPNase	DPN pyrophosphatase
Liver		90. 3	69. 4	21. 3
Kidney		80. 2	63. 5	16. 1
Spleen		128. 6	100. 6	29. 2
Sarcoma 37	6	59. 5	48. 0	13. 7
Liver		80. 6	68. 5	12. 2
Lymphoma #1	14	44. 9	27. 9	17. 1
Liver		90. 3	73. 7	16. 9
Lymphoma #2	12	40. 8	33. 1	8. 6
Liver		105. 0	87. 6	17. 0
Kidney		88. 1	78. 4	9. 4
Spleen		110. 2	81. 0	28. 8
Leukemia L1210	6	61. 0	40. 0	21. 1
Liver		108. 0	81. 9	27. 0
Spleen		68. 5	54. 2	14. 3
H-P melanoma	18	55. 2	41. 0	13. 8
Liver		107. 1	95. 3	10. 7
Krebs-2 carcinoma	7	42. 3	27. 6	14. 8
Liver		92. 2	68. 4	23. 1
Hepatoma 129	14	42. 2	35. 1	8. 4
Liver		104. 1	94. 8	10. 0

*CAF_1 mice were employed in groups containing non-tumor-bearing mice and mice bearing sarcoma 37, lymphomas #1 and #2, Harding-Passey melanoma, and Krebs-2 carcinoma. CDBA hybrids [(BALB/c × DBA/2)F_1] were used for leukemia L1210 and [(C3H × C57BL/6)F_1] were used for hepatoma 129.

†Expressed as μg. DPN split per 0.1 mg. tissue N during incubation for 8 minutes at 37.5° C.

Effect of NCI-3022 on DPNase and DPN-pyrophosphatase activity of sarcoma 37 and of liver.—The effects of administration of 20 μg. per gm. of NCI-3022 on DPNase, DPN pyrophosphatase, and on combined DPN-splitting enzymes of sarcoma 37 and of liver are listed in table 2. Here, apparently, most of the DPNase and DPN-pyrophosphatase activity was retained in the tumor tissues at all intervals after injection, and even after 24 hours most of the activity was recovered. This showed that DPN-splitting enzymes of tumor were unaffected by the action of the drugs even though hemorrhage and necrosis in tumors was brought about by the action of the drug.

TABLE 2.—*Effect of NCI-3022 (20 μg. per gm.) on DPN nucleotidase and DPN-pyrophosphatase of sarcoma 37 and of liver*

Time after injection (*hours*)	Residual activity*†					
	Tumor			Liver		
	Combined (*percent*)	DPNase (*percent*)	DPN pyrophosphatase (*percent*)	Combined (*percent*)	DPNase (*percent*)	DPN pyrophosphatase (*percent*)
2	83	82	93	98	94	108
4	93	91	97	104	107	97
6	106	112	88	107	112	104
24	104	104	94	100	104	94

*$\frac{\text{Activity of the treated}}{\text{Activity of the control}} \times 100$.

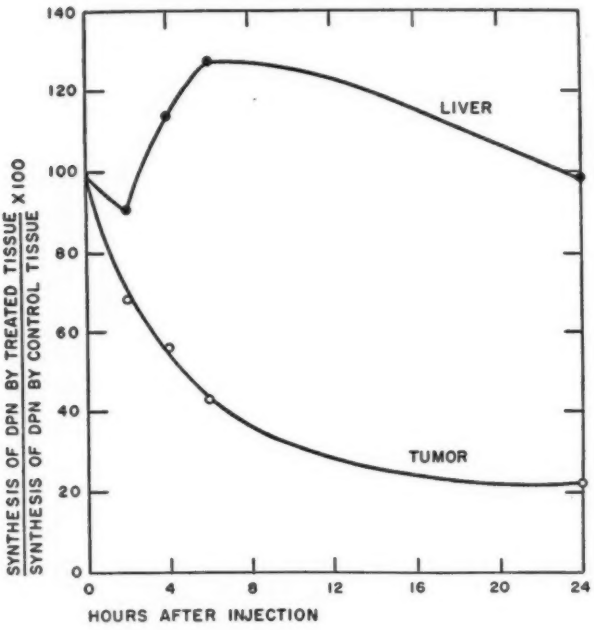
†Range of control values: μg. DPN split per 0.1 mg. N in 8 minutes at 37.5° C.

	Liver	Tumor
Combined	79.7–80.6	59.3–59.5
DPNase	65.6–68.5	46.1–48.0
DPN pyrophosphatase	11.5–12.2	11.9–13.7

Synthesis of DPN by homogenates of sarcoma 37 from animals injected with NCI-3022.—Investigations on the rate of DPN synthesis by the homogenates of tumor were considered next. Text-figure 2 gives the effect of the tumor-damaging compound (NCI-3022 at 20 μg. per gm.) on the synthesis of DPN by sarcoma 37 from drug-treated animals sacrificed at several intervals. The rate of DPN synthesis by the tumor was diminished to about one half of that in the untreatedt umors in about 4 hours. This decrease in rate of DPN synthesis simulated the loss of DPN in treated tumors reported previously (*1*). This appears to be one of the reasons for the lowered level of DPN in the treated tumors.

The homogenates of livers from tumor-bearing mice receiving the injection of the drug showed increased ability to synthesize DPN during this same period of time. While the tumor tissue had only about 40 percent of the activity of the control tumor, the livers from the treated mice exhibited about 130 percent more activity than that of the livers of untreated tumor-bearing animals. Despite this rise in synthetic capacity, the latter still did not attain the normal level.

The homogenates of livers from mice bearing a 6-day-old intramuscular implant of sarcoma 37, in the absence of the drug, showed about a 50 percent decrease in ability to synthesize DPN compared with the liver homogenates from the non-tumor-bearing mice. This led us to investigate further the synthesis of DPN by the livers from mice bearing a tumor each day following tumor transplantation. Text-figure 3 shows the decreased ability to synthesize DPN by the homogenates of livers from mice bearing an intramuscular implant of sarcoma 37. Until about the 4th day following tumor transplantation, the synthesis of DPN by liver homogenates decreases regularly; it then remains steady until at least the 8th day after transplantation. By this time, the level of DPN formed per mg.



TEXT-FIGURE 2.—Synthesis of DPN by homogenates of sarcoma 37 and of liver from mice injected with NCI-3022 (20 μg. per gm.). Range of control values: Micromoles of DPN synthesized per mg. N per hour.

Sarcoma 37	0. 18–0. 19
Liver	0. 24–0. 25

N per hour by liver homogenates from mice bearing 6-day-old tumor transplants was 0.242 μM, as compared with a level of 0.475 μM in liver homogenates from non-tumor-bearing mice.

The synthesis of DPN has been reported as the property of nuclei (*13*), and the breaking up of the nuclei by mechanical means results in a

TABLE 3.—*Effect of sonic vibration on DPN synthesis by homogenates of liver and of sarcoma 37*

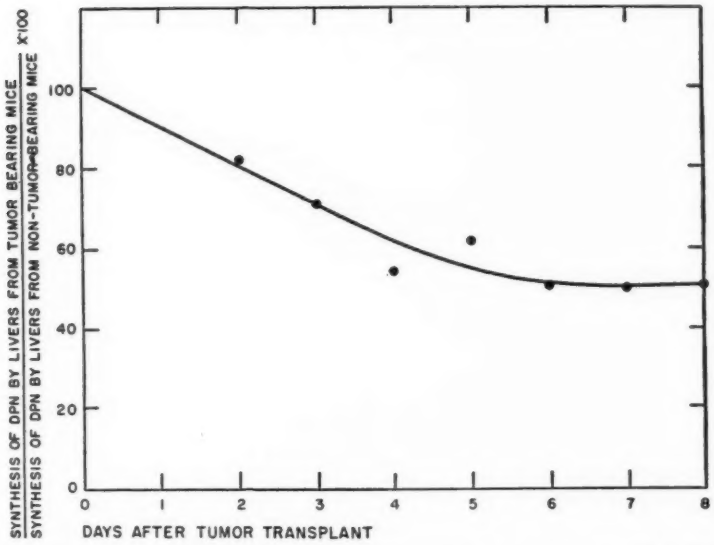
Tissue homogenates *	Synthesis of DPN †		Loss (*percent*)
	Before treatment	After treatment ‡	
Liver	0. 475	0. 285	40
Sarcoma 37	0. 184	0. 084	54

*Homogenates of liver from non-tumor-bearing CAF_1 mice about 2½ months old, and homogenates of tumor from mice bearing 6-day-old sarcoma 37.

†Expressed as micromoles of DPN formed per mg. N per hour.

‡Homogenates were subjected to sonic vibration for 30 minutes in a Raytheon Magnetostriction oscillator, 50 watts, 9 kc., model S-102A, at a plate voltage of 110.

loss in synthesis of DPN (*13*). It is also our experience that the cells subjected to sonic vibrations for 10 minutes at 0° C. suffer approximately a 10 percent decrease in DPN synthesis. A further loss in activity occurred in the tissue homogenates following sonic treatment for half an hour. Table 3 shows the effect of sonic vibrations on the synthesis of DPN by the homogenates of liver and of sarcoma 37. About 40 to 50 percent of the capacity of these homogenates to synthesize DPN was lost.



TEXT-FIGURE 3.—Synthesis of DPN by homogenates of livers from mice bearing intramuscular implants of sarcoma 37 each day after transplantation of the tumor.

Synthesis of DPN by homogenates of liver from nontumorous mice was 0.475 μM DPN formed per mg. N per hour.

In vitro effects of the drug on the DPN-splitting enzymes and on the synthesis of DPN showed that the incubation of the tumor tissue with 2.5 mg. of NCI-3022 per gm. of tissue for a period of 3 hours at 37.5° C. resulted in little or no loss in these enzyme activities.

Discussion

Data presented here show that the activity of DPN-splitting enzymes of the tumors, such as DPNase and DPN pyrophosphatase, was not affected following injection of the drug. However, the rate of synthesis of DPN from NMN in these tumors was markedly affected. This effect was almost parallel to the decreases produced in the DPN level of the treated tumors previously reported (*1*).

The work of Hogeboom and Schneider (*13*) indicates that the synthesis of DPN occurs in the nucleus of the cell, whereas DPN is considered a

component of the cytoplasm. As pointed out by these authors, it is likely that after the synthesis of DPN in the nucleus, DPN is transferred to the cytoplasm. Therefore the present studies show that the effect of the drug is not restricted to the mitochondrial enzymes of the cell, such as cytochrome oxidase activity and succinoxidase activity, but extends also to the other systems of enzymes belonging to the nuclear part of the cell.

It has been reported previously (*15*) that the cells of sarcoma 37 showed shrinkage of nuclei and retraction of the cytoplasm soon after the administration of the drug. After 6 to 8 hours further degenerative changes occurred; nuclei were pyknotic and the cytoplasmic material showed extensive retraction. With such marked damage induced in the tumors by the drug, it is possible that some of the synthetic processes in these tumor cells are affected. Therefore reduced ability to synthesize DPN by tumor homogenate from drug-treated animals, as reported here, might be expected because of the damage to the nuclei. However, it is important to fortify such a statement by following the effect of the drug on the biosynthesis of DPN by isolated nuclei of tumor cells from animals receiving the drug. According to the previously cited authors (*13*), certain difficulties are encountered in fractionating cellular material to recover all the enzyme activity in the isolated nuclei. These difficulties involve breakage of nuclei during the process of fractionation, with the result that some of the enzyme activity is recovered in the supernatant fraction. In the case of tumors from drug-treated animals, the tumor cells are markedly damaged and therefore the chances of isolation of all the intact nuclei from such tissues are remote.

We have observed (*16*) that the drug-induced changes in the cytochrome oxidase and succinoxidase activity of tumors are more pronounced than those produced by physical means. Similarly, the present report shows that the disruption of cellular material by physical means does not bring about as marked a loss in the enzyme promoting DPN synthesis as in the enzyme activity of the damaged tumors.

Reduced activity of the DPN-synthesizing enzyme of the livers from tumor-bearing mice is an interesting observation from the point of view of changes brought about by the introduction of a tumor implant in the body of the animal, which seems to upset the normal function of the liver. This phenomenon is reminiscent of the observations of Greenstein and his associates on liver catalase (*17*). Unlike catalase (*18,19*), however, DPN-synthesizing enzyme was found in measurable quantities in both tumor and liver, and the changes in enzymes in both types of tissue could be followed.

The effect of intramuscular transplants of other tumors on the DPN-synthesizing enzymes in liver is being investigated. It is conceivable that these changes may reflect the role of synthetic processes of tumors affecting similar synthetic processes in normal tissues. It would be of interest to see what effects removal of the tumor by excision or spontaneous regression of tumors would have on the DPN-synthesizing enzyme of liver.

Despite the fact that the decrease in endogenous DPN can be accounted for by the loss in ability of homogenates to synthesize DPN, it is also conceivable that when the cells are damaged the DPNase, while unchanged in its activity, might be released in a form which would enable it to act more effectively on the endogenous DPN. This might explain the need to fortify the substrate with a large excess of DPN so that tumors from treated animals might be able to utilize glucose at the same rate as found in tissues from untreated animals (*1*). This aspect is being explored further.

Summary

1) The diphosphopyridine nucleotidase and diphosphopyridine nucleotide-pyrophosphatase activities of various transplanted tumors in mice have been determined.

2) The DPNase and DPN-pyrophosphatase activity of homogenates of sarcoma 37 and of liver from tumor-bearing mice receiving injections of 20 μg. per gm. of acetylpodophyllotoxin-ω-pyridinium chloride showed rather small changes in enzyme activity compared with those in similar tissues from animals not treated with the drug.

3) The ability of tumor homogenates from the treated animals to synthesize DPN was markedly reduced, while the capacity for synthesis of DPN from nicotinamide mono-nucleotide by the homogenates of livers from these animals increased proportionately to the damage induced in the tumors.

4) Disruption of liver cells from non-tumor-bearing mice and of tumor cells by physical means resulted in a 50 percent loss in ability of the enzyme in these tissues to synthesize DPN.

5) Liver homogenates from tumor-bearing mice showed approximately 50 percent less activity for synthesis of DPN than the homogenates of the livers from non-tumor-bearing mice.

References

(*1*) Waravdekar, V. S., Paradis, A. D., and Leiter, J.: Enzyme changes induce in normal and malignant tissues with chemical agents. IV. Effect of *alpha*-peltatin on glucose utilization by sarcoma 37 and on the adenosinetriphosphatase, hexokinase, aldolase, and pyridine nucleotide levels of sarcoma 37. J. Nat. Cancer Inst. 16: 31–39, 1955.

(*2*) Handler, P., and Klein, J. R.: The inactivation of pyridine nucleotide by animal tissues *in vitro*. J. Biol. Chem. 143: 49–57, 1942.

(*3*) Kornberg, A., and Lindberg, O.: Diphosphopyridine nucleotide pyrophosphatase. J. Biol. Chem. 176: 665–677, 1948.

(*4*) Schrecker, A. W., Greenberg, G. Y., and Hartwell, J. L.: Components of podophyllotoxin. XVI. Podophyllotoxin haloacetates and quaternary derivatives. J. Am. Chem. Soc. 76: 1184–1185, 1954.

(*5*) Waravdekar, V. S., Domingue, A., and Leiter, J.: Effect of podophyllotoxin, *alpha*-peltatin and *beta*-peltatin on the cytochrome oxidase activity of sarcoma 37. J. Nat. Cancer Inst. 13: 393–407, 1952.

(*6*) Leiter, J., Paradis, A. D., and Waravdekar, V. S.: Enzyme changes induced in normal and malignant tissues with chemical agents. II. Effects of various compounds on the cytochrome oxidase activity of transplanted tumors. J. Nat. Cancer Inst. 14: 177–188, 1953.

(7) COLOWICK, S. P., KAPLAN, N. O., and CIOTTI, M. M.: The reaction of pyridine nucleotide with cyanide and its analytical use. J. Biol. Chem. 191: 447–459, 1951.

(8) KAPLAN, N. O., COLOWICK, S. P., and NASON, A.: Neurospora diphosphopyridine nucleotidase. J. Biol. Chem. 191: 473–483, 1951.

(9) MANN, P. J. G., and QUASTEL, J. H.: Nicotinamide, cozymase and tissue metabolism. Biochem. J. 35: 502–517, 1941.

(10) RACKER, E.: Crystalline alcohol dehydrogenase from bakers' yeast. J. Biol. Chem. 184: 313–319, 1950.

(11) OHLMEYER, P.: Darstellung von reiner Dihydrocozymase in Substanz. Biochem. Ztschr. 297: 66–69, 1938.

(12) KORNBERG, A.: Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. J. Biol. Chem. 182: 779–793, 1950.

(13) HOGEBOOM, G. H., and SCHNEIDER, W. C.: Cytochemical studies. VI. The synthesis of diphosphopyridine nucleotide by liver cell nuclei. J. Biol. Chem. 197: 611–620, 1952.

(14) MA, T. A., and ZUAZAGA, G.: Micro-Kjeldahl determination of nitrogen. Ind. and Eng. Chem., anal. ed. 14: 280–282, 1942.

(15) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in sarcoma 37 with podophyllin, podophyllotoxin alpha-peltatin, beta-peltatin, and quercetin. J. Nat. Cancer Inst. 10: 1273–1293, 1950.

(16) WARAVDEKAR, V. S., PARADIS, A. D., and LEITER, J.: Enzyme changes induced in normal and malignant tissues with chemical agents. V. Effect of acetylpodophyllotoxin-ω-pyridinium chloride on uricase, adenosine deaminase, nucleoside phosphorylase, and glutamic dehydrogenase activities. J. Nat. Cancer Inst. 16: 99–105, 1955.

(17) GREENSTEIN, J. P., JENRETTE, W. V., and WHITE, J.: The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. J. Nat. Cancer Inst. 2: 283–291, 1941.

(18) GREENSTEIN, J. P., JENRETTE, W. V., MIDER, G. B., and ANDERVONT, H. B.: The relative enzymatic activity of certain mouse tumors and normal control tissues. J. Nat. Cancer Inst. 2: 293–299, 1941.

(19) GREENSTEIN, J. P.: Further studies of the liver catalase activity of tumor-bearing animals. J. Nat. Cancer Inst. 3: 397–404, 1943.

The Probability of Developing Cancer[1]

IRVING D. GOLDBERG, *M. P. H.*, MORTON L. LEVIN, *M. D.*, PAUL R. GERHARDT, *M. D.*, VINCENT H. HANDY, *M. D.*, *and* RITA E. CASHMAN, *M. S.*,[2]
Division of Medical Services, New York State Department of Health, Albany, New York

It has only been within the last 20 years that reasonably reliable statistics have become available regarding the incidence of cancer in man. Up until that time, mortality data were used almost exclusively to estimate and describe the occurrence of this disease. In Connecticut, reports on cancer morbidity have been gathered from hospitals and tumor clinics on a voluntary basis since 1935 (*1*), and in New York State, exclusive of New York City, cancer has been a reportable disease by law since 1940. Variations in the case fatality and natural history of different forms of cancer, which emphasize the need for reliable morbidity statistics, are evident in a comparison of mortality and incidence of cancer of various sites. Skin cancer, for example, is generally recognized as the most frequently occurring type of cancer in this country, but the ease with which it is cured places it far down the list in cancer mortality. Breast cancer among females, on the other hand, is very high on the frequency lists of both incidence and mortality (*2,3*).

The availability of cancer-morbidity data has made possible the calculation of another useful index of cancer risk, that is, the probability of developing cancer throughout the remaining lifetime of persons of various ages. Data on cancer incidence and mortality do not *per se* describe this risk. A preliminary study of this question, based on cancer incidence in New York State, exclusive of New York City (hereafter referred to as upstate New York) was reported at the Fourth International Cancer Research Congress (*4*). The present paper is based on more complete data and a number of modifications of methodology.

In the present study, the probability of developing cancer throughout life was calculated for total cancer and for each of over 25 specific sites, separately for males and females. The study is subdivided into two 3-year periods (1942–44 and 1949–51) to determine any trend. This report is concerned with a description of the method used in the calculation of the probabilities, as well as a discussion of the results.

---

[1] Received for publication March 26, 1956.
[2] Grateful acknowledgment is made to Miss Kathryn C. Coddington, of the New York State Department of Health, for her assistance in the calculation of the probabilities.

Source Material

Because both the methodology and interpretation of the results are influenced by the nature of the basic data, a description of the way in which cancer-morbidity statistics are collected and enumerated in upstate New York seems desirable.

Effective January 1, 1940, a legislative act made it mandatory for all physicians, hospitals, and laboratories in upstate New York to report all cases of cancer coming to their attention. As indicated by Levin (*5*), cancer reporting has had the continued support of the physicians of the State. Cases are reported to the State Bureau of Cancer Control, through the local health office, on a report card furnished by the Department or on a laboratory report form. Each report received in the central office is checked against a master index file to determine whether or not the case has ever been previously reported for any cancer site. Cases found to have been reported previously (in the same or an earlier year), *even if for a different site of cancer*, are considered as duplicate reports, while those reported for the first time are termed new cases. In addition, the New York State Office of Vital Statistics routinely advises the Bureau of Cancer Control of all death certificates on which cancer is mentioned either as the primary or contributing cause of death. These deaths are also checked against the master index file, and all deaths never previously reported ("unreported deaths") are treated as new cases. Thus, the "unreported deaths" plus the cases reported for the first time while alive make up the total of new cases. These new cases, which describe the incidence of cancer during any year, form the basis on which the probabilities for developing cancer were calculated.

The adequacy of any system of morbidity reporting depends in the first instance on the completeness of reporting. Since clinical cancer is a progressive disease, cancer cases undiagnosed in any given year should contribute very little to the understatement of morbidity. These cases would appear in the statistics for new case reports—through case report or by death certificate—in the year in which they are eventually reported; and there should be a counterbalancing effect over the years. With respect to reporting of known cases, a joint study by the United States Public Health Service and the New York State Department of Health found that for cancer cases known to hospitals, tumor clinics, and laboratories in 1945, more than 80 percent were reported to the Bureau of Cancer Control.[3] When one considers that a large proportion of cases unreported while alive eventually will be reported by death certificates, the incompleteness of the morbidity data should be appreciably less than 20 percent.[4] Due to the difficulties involved in estimating and correcting for this incompleteness, particularly by site of disease, no adjustments were

[3] While the completeness of reporting of cases known to private physicians was not studied, it is reasonable to assume that a great majority of these cases also become known to, and are reported by, hospitals, tumor clinics, and laboratories, in addition to those otherwise reported by the private physician or by death certificate.

[4] When checked against all 1945 and 1946 case and death reports, between 84 and 96 percent of the cases of cancer of each of the major-site groups, diagnosed in 1945 and given hospital or tumor-clinic service in that year, had been reported.

made for this factor. Also no reliable estimate of cases never diagnosed is available. Thus, the term "probability of *developing* cancer" for any or all sites, as used in this report, means the probability of *being reported* with cancer for the first time, regardless of site.

The two most important factors for which allowance had to be made in the methodology were: 1) the fact that all deaths with cancer stated as a cause on the death certificate are included in the incidence rates, either as "unreported deaths" or as cases reported by card or laboratory report, and 2) the fact that once an individual has been reported for cancer of any site, that individual is never again considered as a new case. The latter factor requires further discussion with regard to its effect on the interpretation of the results.

There have been many studies reported (*6–13*) on the tendency for increased or lessened susceptibility to a second primary tumor among persons with cancer of the same or a different organ. Both ends of the scale have been reported: Peller (*9,10*) concluding that a cured cancer serves to protect against development of other malignant neoplasms, while Lombard *et al.* (*12*) suggest that persons with skin cancers are predisposed to other skin cancers, and Mider *et al.* (*13*) conclude that subjects with carcinomas of the breast or large intestine ". . . have a significantly increased probability of developing a second cancer of the same or similar histogenesis." It is not the purpose here to evaluate the reported findings but rather to point out that differences may exist between the incidence rate of initial and second primary malignancies. Due to the way in which the morbidity data in upstate New York are gathered and recorded it was not feasible to take multiple tumors into account, so that the probability of developing cancer as defined in this report describes the risk to an individual of being reported with a first primary malignancy.

According to the 1950 Census of Population, less than 3 percent of the upstate New York population was nonwhite. Thus, while no consideration was given to race, the results will be those for a predominantly white population. As mentioned earlier, the periods of study were 1942–44 and 1949–51. The latter period was chosen because of the applicability of the 1950 population census for reliable rates. While reporting of cancer cases began in 1940, the first 2 years of reporting apparently included a backlog of "old" cases. The level of cancer reporting did not stabilize until 1942, so that the 3-year period of 1942–44 represents the earliest period which was considered comparable to that of the 1949–51 period.

Method

Basic abridged life-table methods[5] were employed in the present study. In the usual life table, by assuming that a fixed number of births—for convenience 100,000—will be subject throughout life to a given set of age-specific mortality rates, the number of deaths (and consequently the

[5] A discussion of life-table construction can be found in Reed and Merrell's "A Short Method for Constructing an Abridged Life Table" (*14*) and in Dublin, Lotka, and Spiegelman's *Length of Life* (*15*).

number remaining alive) at various ages is computed. By the same reasoning, we can assume that a fixed set of cancer-morbidity rates will prevail over the same period and calculate how many persons will develop cancer.

To obtain the probability of developing cancer, it is necessary to estimate for the life-table population not only the number of cancer cases developing between ages x and $x + n$, but also the number of survivors at age x who had not previously acquired the disease, which would be the population at risk of developing the disease for the first time in the next age interval.

The exact procedure used was as follows:

The 1939–41 and 1949–51 life tables for upstate New York were constructed by the Office of Vital Statistics of the State Health Department utilizing the method described by Reed and Merrell (*14*). Each life table was based on the mortality rates for the period described by the life table. The age-specific mortality rates from all causes, ${}_nm_x$, were calculated by dividing the annual average number of deaths during the period by the population at the middle of the period. For 1939–41 the population at the 1940 census was used and for 1949–51 the 1950 census population was employed.

The life tables provided the following columns which were utilized in the calculation of the cancer probabilities:

${}_nL_x$, the life-table population in the age group x to $x + n$;

l_x, the number of persons surviving to exact age x out of the cohort of 100,000 live births ($l_o = 100{,}000$);

${}_nq_x$, the probability of dying in the age group x to $x + n$;

${}_nd_x$, the number of deaths in the age group x to $x + n$ from among the survivors to exact age x (l_x).

The age-specific cancer-morbidity rates were computed by dividing the annual average number of new cases reported during the period by the population at the middle of the period. For 1949–51 the population at the 1950 census was used; for 1942–44, estimates of the population as of July 1, 1943. These rates were designated I_x for total cancer and I_x^s for cancer of a specific site.

The number of cases expected to occur in the life-table population between ages x and $x + 5$ was obtained by taking the life-table population ${}_5L_x$ and multiplying it by the incidence rate for the corresponding age group. That is, omitting 5 (designating the 5-year age interval) from the subscripts,

$$c_x = I_x L_x$$

and for specific sites,

$$c_x^s = I_x^s L_x.$$

The number of cancer cases expected to occur after age x was obtained by summing the expected number of cases in the age group x to $x + 5$ and all subsequent age groups. Thus, for all forms of cancer,

$$t = \sum_{x}^{\infty} c_x,$$

so that the number of cases expected to occur from birth on is the sum of the entire c_x column. Likewise, the cases expected to occur between any two ages (given in the life table) is the sum of the cases in the c_x column between those ages. The situation is similar for specific sites of the disease except that t_x is replaced by t_x^s where

$$t_x^s = \sum_{x}^{\infty} c_x^s.$$

The problem is to divide the surviving population, l_x, into survivors with cancer, l_x^c, and survivors without cancer, l_x'. It is assumed that at birth the population is cancer-free ($l_o^c = 0$). The population with cancer at age 5 will equal the number of cancer cases reported before age 5 minus deaths among these cases. Similarly, the survivors without cancer will be the births (l_o) diminished by cancer cases reported before age 5 (which will include all cancer deaths) and by all other deaths.

The life-table deaths, d_x, and probability of dying, q_x, can be divided into deaths and probability of dying from cancer (d_x^c, q_x^c) and all other causes (d_x', q_x'), assuming that

$$\frac{d_x^c}{d_x} = \frac{m_x^c}{m_x},$$

where m_x is the observed death rate from all causes upon which the life table was based and m_x^c the observed death rate from cancer for the same population over the same time period.

Then,

$$q_x^c = \frac{d_x^c}{l_x} \qquad \text{where } d_x^c = \frac{m_x^c d_x}{m_x}$$

and

$$q_x' = \frac{d_x'}{l_x} \qquad \text{where } d_x' = d_x - d_x^c.$$

However, d_x' is not the number of deaths among persons who have never developed cancer; it includes non-cancer deaths among persons who have been reported with cancer at some time previously. Likewise, d_x^c is not the total number of deaths among persons who have been reported with cancer at any time previously; it does not include deaths of these persons from causes other than cancer. If we assume that the cancer population has the same risk of death from other causes as the total population $\left(q_x' = \frac{d_x'}{l_x}\right)$, the non-cancer deaths among persons previously reported with cancer can be estimated by applying the probability of dying from other causes to the effective cancer population exposed to this risk. Let us assume that the new cancer cases and the cancer deaths in the age span x to $x + n$ occur uniformly during the interval, so that on the average they occur halfway through the period. Then, the new cases entering the cancer cohort in the interval are subject to the risk of dying

from other causes for only half the period (equivalently, half the group is subject to the risk for the entire period). Also, the individuals who die from cancer in the age span may be considered as withdrawals "alive," insofar as the risk of death from other causes is concerned, and they too are subject to this latter risk for only half the period. Thus, the effective cancer population exposed to the risk of dying from causes other than cancer becomes $l_x^c + \frac{1}{2}c_x - \frac{1}{2}d_x^c$; and the estimated non-cancer deaths among this population are obtained by applying q_x' to this effective population.

If we designate these non-cancer deaths of the cancer population, in the interval x to $x + n$, as K_x,

where $$K_x = q_x' (l_x^c + \tfrac{1}{2}c_x - \tfrac{1}{2}d_x^c),$$

then $$l_{x+n}^c = l_x^c + c_x - d_x^c - K_x$$

and $$l_{x+n}' = l_x' - c_x - d_x' + K_x.$$

Thus, by assuming the population is cancer-free at birth, so that $l_0^c = 0$ and $l_0' = 100{,}000$, the survivors to age 5 with cancer (l_5^c) and the survivors without cancer (l_5') are calculated from these equations. The procedure is then repeated for each of the succeeding age groups.

The probability of eventually developing any form of the disease after exact age x is obtained by dividing t_x (the cumulated cases in the life table after age x) by l_x'. At birth, $l_0' = l_0$ so that the probability of developing cancer from birth on becomes t_0 (the total cancer cases in the life table) divided by 100,000. The probability of developing cancer between any two ages is obtained by dividing the number of cases occurring between those ages by the "cancer-free" population alive at the earlier age. Between 40 and 60 years this becomes $(t_{40} - t_{60}) \div l_{40}'$. For specific sites of cancer, the same procedure obtains except, of course, that the appropriate t_x' column for the site in question is used.

The procedures discussed above were used in the calculation of the probability of developing cancer, of all forms and for selected sites, by sex, for the two periods 1949–51 and 1942–44. For the period 1949–51, the cancer-incidence rates prevailing at that time were applied to the 1949–51 life-table population for upstate New York. No life table for upstate New York was available for 1942–44 so that the incidence rates for that period were applied to the 1939–41 upstate life-table population. While this modification should not have any appreciable effect, the calculated probabilities for 1942–44 may be interpreted as probabilities adjusted to the mortality conditions of 1939–41.

Results

As was noted in the procedure for calculating the cancer probabilities, the population at risk of developing the disease was largely dependent on the death rate (and therefore the probability of dying) from non-cancer causes throughout the life span. Before the results of the study are

presented, it would, therefore, be well to evaluate the effect of deaths from non-cancer causes on the probability of developing cancer. This is of particular interest in the comparison of the probabilities for different periods of time (as here, 1942–44 *vs.* 1949–51).

The question to be considered is: How much does the risk of dying from non-cancer causes affect the probability of developing cancer?—or phrased in another way: How much of the difference between the periods 1942–44 and 1949–51 in the calculated probabilities of developing cancer is due to changes in the non-cancer death rate and how much is due to the change in cancer morbidity?

To answer this question the adjusted probability of developing cancer from birth on was calculated for several sites by applying the prevailing incidence rates for the 1949–51 period to the stationary life-table population (L_x) in 1939–41 for upstate New York. Summing the cases so obtained (for each age) over the entire life table and dividing this sum by the original cohort of 100,000 live births gives the adjusted probability of developing cancer from birth on. The difference in this adjusted probability and that already calculated based on the 1949–51 life-table population measures the effect of changing mortality conditions from non-cancer causes between the 1939–41 and 1949–51 periods. Likewise, the difference between the adjusted 1949–51 probability and that for 1942–44 (which was also based on the 1939–41 life table) describes the effect of change in incidence rates between 1942–44 and 1949–51.

A comparison of the 1949–51 probabilities of developing cancer from birth on for several sites with the probabilities for 1942–44 and 1949–51 adjusted to the 1939–41 life table appears in table 1. The results clearly indicate that the observed change in the probability of developing cancer over a period of time is not due solely to the force of cancer morbidity, but is considerably influenced by changes in non-cancer mortality. Thus, the general decline from 1939–41 to 1949–51 in the non-cancer mortality rate accounted for about 40 percent of the observed increase in the probability of eventually developing cancer (all forms) among males and for almost two thirds of the increase observed among females. For cancer of the stomach, in both males and females, the adjusted 1949–51 probability reversed the direction of change between 1942–44 and 1949–51 from an increase to a decrease. Further, the reported stomach-cancer incidence rate for males in 1942–44 was slightly lower than in 1949–51 (23.6 per 100,000 as compared with 23.8), while the reverse was true for stomach cancer among females (15.0 *vs.* 13.0). It is evident that a falling or rising cancer-incidence rate does not necessarily imply a trend in the probability of developing cancer in the same direction. Rather, the apparent paradox of a declining (or rising) cancer-incidence rate and an increasing (or falling) probability of developing the disease is a distinct possibility.

In presenting the results for both study periods, no further attempt will be made to explain any differences in terms of changing non-cancer mortality conditions. Rather, the results as presented for 1942–44 and 1949–51 will be those based on the 1939–41 and 1949–51 life tables, respectively,

for New York State, exclusive of New York City. It is sufficient to appreciate that any observed differences are due to the combined effect of the forces of cancer morbidity and mortality from other causes.

As was mentioned earlier, the probability of developing cancer was calculated for over 25 selected sites, by sex, for 1942–44 and 1949–51. There are some relatively very minor differences between 1942–44 and 1949–51 in the classification of sites which, for practical purposes, can be ignored; so that the level of probability for a given site in the earlier period can be compared directly with that for the 1949–51 period. It should be pointed out, however, that factors such as improved diagnosis over the 7-year span may contribute to apparent differences. For example, it is believed that more accurate site diagnosis may have ac-

TABLE 1.—*Probability of developing cancer from birth on for leading sites by sex; New York State, exclusive of New York City, 1942–44, 1949–51, and 1949–51 adjusted*

Sex and site	Probability (*in percent*)		
	1942–44*	1949–51	1949–51 adjusted†
Male			
All sites	15.636	21.572	19.151
Skin	2.111	2.988	2.646
Prostate	1.759	2.613	2.219
Lung and bronchus	0.918	2.114	1.926
Stomach	1.784	1.994	1.761
Intestines	1.290	1.894	1.673
Female			
All sites	19.491	24.990	21.405
Breast	4.390	5.544	4.792
Intestines	1.764	2.691	2.247
Skin	1.536	2.507	2.102
Cervix	2.030	2.319	2.093
Fundus uteri	1.188	1.600	1.414
Stomach	1.317	1.348	1.092
Lung and bronchus	0.222	0.369	0.310

*Based on 1942–44 cancer-incidence rates applied to 1939–41 life-table populations for New York State, exclusive of New York City.

†Adjusted to 1939–41 life-table populations for New York State, exclusive of New York City.

counted for some of the rise observed in the incidence rate for intestinal cancer and the concurrent drop in the observed incidence rate for cancer of the stomach. How much of this factor is reflected in the observed changes in probability would be a matter of conjecture.

A summary of the results is contained in tables 2 through 7. The probability of developing cancer from birth on for each of the selected sites and for total cancer, by sex, is given in table 2 for the 1949–51 period and in table 3 for the 1942–44 period. Where a site is listed in the table for one study period and not the other, this is because data for that site were not available in both study periods. Tables 4 and 5 give the 1949–51 site-specific probabilities of eventually developing cancer at selected ages for males and females, respectively. Tables 6 and 7, which are based on 1949–51 data for males and females, respectively, provide the information

TABLE 2.—*Probability of developing cancer from birth on by sex and site based on cancer-morbidity reports, New York State, exclusive of New York City, 1949–51*

Male		Female	
Site	Probability (*in percent*)	Site	Probability (*in percent*)
Skin	2. 988	Breast	5. 544
Prostate	2. 613	Intestines	2. 691
Lung and bronchus	2. 114	Large	(2. 650)
Stomach	1. 994	Small	(0. 041)
Intestines	1. 894	Skin	2. 507
Large	(1. 851)	Cervix	2. 319
Small	(0. 043)	Fundus uteri	1. 600
Rectum	1. 224	Stomach	1. 348
Bladder	1. 140	Rectum	1. 016
Leukemia and aleukemia	0. 622	Ovary	0. 993
Pancreas	0. 618	Pancreas	0. 530
Lip	0. 486	Bladder	0. 528
Esophagus	0. 476	Leukemia and aleukemia	0. 508
Larynx	0. 402	Lung and bronchus	0. 369
Kidney	0. 324	Biliary passages	0. 360
Liver	0. 267	Liver	0. 302
Mouth	0. 260	Kidney	0. 236
Brain	0. 243	Rectosigmoid	0. 224
Tongue	0. 239	Lymphosarcoma	0. 206
Rectosigmoid	0. 222	Vulva	0. 199
Lymphosarcoma	0. 218	Hodgkin's disease	0. 173
Hodgkin's disease	0. 210	Thyroid gland	0. 166
Testis	0. 159	Brain	0. 163
Biliary passages	0. 142	Esophagus	0. 146
Pharynx	0. 127	Salivary gland	0. 113
Bone	0. 110	Bone	0. 093
Salivary gland	0. 084	Mouth	0. 080
Thyroid gland	0. 068	Tongue	0. 070
Breast	0. 049	Lip	0. 040
		Larynx	0. 034
		Pharynx	0. 032
All others	2. 278	All others	2. 399
All sites	21. 572	All sites	24. 990

necessary to calculate the probability of developing cancer between any two indicated ages for each of the leading sites.

While no attempt will be made to compare all the findings, some results merit further comment. In either study period, the probability of developing cancer (all forms) from birth on among females was appreciably higher than that for males. In the 1942–44 period the eventual cancer risk after birth among females was 25 percent higher than that for males (0.195 *vs.* 0.156), while for the 1949–51 period this decreased to 12 percent (0.250 *vs.* 0.216). Further, the continued trend of the female incidence rate and the more rapidly rising incidence rate for males [since 1949–51 the rate for males has surpassed that for females (*16*)] suggest that the probability of developing cancer among males may eventually exceed that for females. According to the results for 1949–51, 25.0 percent of females—or 1 in every 4 female births—may be expected to develop cancer sometime during her life span, while for males 21.6 percent—or more than 1 in 5 male births—can be expected to develop

TABLE 3.—*Probability of developing cancer from birth on by sex and site based on cancer-morbidity reports, New York State, exclusive of New York City, 1942–44*

Male		Female	
Site	Probability (*in percent*)	Site	Probability (*in percent*)
Skin	2. 111	Breast	4. 390
Stomach	1. 784	Cervix	2. 030
Prostate	1. 759	Intestines	1. 764
Intestines	1. 290	Skin	1. 536
Lung and bronchus	0. 918	Stomach	1. 317
Rectum	0. 901	Fundus uteri	1. 188
Bladder	0. 800	Ovary	0. 799
Lip	0. 491	Rectum	0. 761
Leukemia and aleukemia	0. 370	Bladder	0. 396
Pancreas	0. 363	Liver	0. 394
Esophagus	0. 327	Leukemia and aleukemia	0. 331
Liver	0. 294	Pancreas	0. 329
Larynx	0. 270	Biliary passages	0. 270
Brain	0. 249	Lung and bronchus	0. 222
Mouth	0. 231	Vulva	0. 176
Kidney	0. 214	Brain	0. 175
Tongue	0. 173	Kidney	0. 164
Hodgkin's disease	0. 151	Rectosigmoid	0. 128
Bone	0. 142	Bone	0. 128
Rectosigmoid	0. 140	Thyroid gland	0. 119
Testis	0. 116	Esophagus	0. 113
Biliary passages	0. 104	Hodgkin's disease	0. 100
Pharynx	0. 083	Mouth	0. 078
Breast	0. 052	Tongue	0. 044
Thyroid gland	0. 029	Lip	0. 036
		Pharynx	0. 032
		Larynx	0. 030
All others	2. 272	All others	2. 444
All sites	15. 636	All sites	19. 491

some form of the disease after birth. The effect of the more rapidly rising incidence rate among males than among females is manifest in the relative rise between the two study periods of 38 percent in the probability of developing cancer for males (from 0.156 to 0.216), as compared with the relative increase of only 28 percent among females (from 0.195 to 0.250).

Of particular note among males was the sharp rise in the probability of developing lung cancer. The risk of developing this form of the disease (from birth on) more than doubled over the short span of 7 years (from 0.009 to 0.021), and this was approximately twice the corresponding relative increase among females (from 0.0022 to 0.0037). With regard to the other sites of cancer, the rise from 1942–44 to 1949–51 in the probability of developing the disease among males was rather general, with only a few rather small decreases being observed. However, more than half of the total cancer risk after birth among males in the 1949–51 period was accounted for by only 5 sites: skin, prostate, lung, stomach, and intestines.

Among females, by far the outstanding observation pertains to breast cancer. This form of the disease is responsible for more than one fifth (22 percent) of the total cancer risk to women (1949–51). As was true

for males, the rise in the probability of developing cancer from birth on was manifest for the very great majority of sites among females. More than 50 percent of the total cancer risk to females, in the 1949–51 period, was confined to malignancies of the breast, intestines, skin, and cervix uteri.

As is evident from tables 4 and 5, the risk of eventually developing cancer is not necessarily greatest at birth, but more usually reaches a maximum at some much later period of life. This is due to the relatively greater cancer incidence during the older ages. However, for some sites or types of malignancy (leukemia, brain, Hodgkin's disease, and lymphosarcomas) the age of maximum eventual risk is at birth or in the first 5 or 10 years of life when the cancer incidence for those sites is relatively high. In any event, the age at which one is confronted with the greatest eventual risk of developing cancer varies for different sites. For example, among males for the 1949–51 period, the risk of developing leukemia was greatest from birth on, while for prostatic cancer the maximum eventual risk was reached at 75 years of age. From the results for 1949–51, females reach the maximum eventual risk of developing some

TABLE 4.—*Probability of eventually developing cancer among males by age and site based on cancer-morbidity reports, New York State, exclusive of New York City, 1949–51*

Site	Eventual probability (*in percent*) at:			
	Birth	Age 20	Age 45	Age 65
Skin	2. 988	3. 124	3. 077	2. 766
Prostate	2. 613	2. 737	2. 923	3. 650
Lung and bronchus	2. 114	2. 214	2. 287	1. 505
Stomach	1. 994	2. 088	2. 163	2. 166
Intestines	1. 894	1. 983	2. 034	1. 946
Large	(1. 851)	(1. 938)	(1. 990)	(1. 906)
Small	(0. 043)	(0. 045)	(0. 044)	(0. 040)
Rectum	1. 224	1. 281	1. 316	1. 245
Bladder	1. 140	1. 192	1. 242	1. 243
Leukemia and aleukemia	0. 622	0. 571	0. 530	0. 474
Pancreas	0. 618	0. 647	0. 671	0. 619
Lip	0. 486	0. 509	0. 509	0. 468
Esophagus	0. 476	0. 499	0. 528	0. 495
Larynx	0. 402	0. 421	0. 437	0. 334
Kidney	0. 324	0. 325	0. 325	0. 216
Liver	0. 267	0. 278	0. 286	0. 266
Mouth	0. 260	0. 271	0. 278	0. 258
Brain	0. 243	0. 224	0. 171	0. 045
Tongue	0. 239	0. 250	0. 260	0. 233
Rectosigmoid	0. 222	0. 232	0. 239	0. 218
Lymphosarcoma	0. 218	0. 221	0. 194	0. 130
Hodgkin's disease	0. 210	0. 203	0. 122	0. 079
Testis	0. 159	0. 163	0. 062	0. 033
Biliary passages	0. 142	0. 148	0. 154	0. 147
Pharynx	0. 127	0. 131	0. 126	0. 091
Bone	0. 110	0. 102	0. 092	0. 087
Salivary gland	0. 084	0. 084	0. 067	0. 048
Thyroid gland	0. 068	0. 068	0. 057	0. 041
Breast	0. 049	0. 051	0. 053	0. 042
All other sites	2. 278	2. 327	2. 270	1. 890
All sites	21. 572	22. 342	22. 471	20. 735

form of cancer at 20 years of age, while for males this peak in eventual risk is not reached until 25 years later.

Quite naturally, some of the general conclusions to be drawn from the probability data are already manifest in the cancer-incidence or mortality statistics alone. For example, the rise in lung cancer among men (and women for that matter) and the narrowing gap in the converging trends of male and female cancer risk are also evident in the morbidity data (*16*). This, of course, is to be expected, for the level of probability or risk is dependent in part on the level of the incidence rates. The order of sites according to degree of risk will be essentially the same as the relative position in terms of incidence rates. Variations may also occur due to factors already mentioned, such as the effect of non-cancer mortality in the calculation of the probability of developing cancer. However, there is little question that the probability data provide an additional useful index in the evaluation of the cancer problem. They provide a measure of the cancer risk to which people are subject—not only from birth or any other age on, but also between any two ages.

TABLE 5.—*Probability of eventually developing cancer among females, by age and site, based on cancer-morbidity reports, New York State, exclusive of New York City, 1949–51*

Site	Eventual probability (*in percent*) at:			
	Birth	Age 20	Age 45	Age 65
Breast	5. 544	5. 732	5. 350	3. 880
Intestines	2. 691	2. 775	2. 783	2. 433
Large	(2. 650)	(2. 733)	(2. 740)	(2. 395)
Small	(0. 041)	(0. 042)	(0. 043)	(0. 038)
Skin	2. 507	2. 578	2. 492	2. 138
Cervix	2. 319	2. 398	1. 912	1. 001
Fundus uteri	1. 600	1. 653	1. 630	0. 935
Stomach	1. 348	1. 394	1. 434	1. 426
Rectum	1. 016	1. 048	1. 053	0. 926
Ovary	0. 993	1. 024	0. 945	0. 552
Pancreas	0. 530	0. 548	0. 568	0. 521
Bladder	0. 528	0. 545	0. 569	0. 576
Leukemia and aleukemia	0. 508	0. 457	0. 413	0. 348
Lung and bronchus	0. 369	0. 381	0. 385	0. 326
Biliary passages	0. 360	0. 372	0. 391	0. 332
Liver	0. 302	0. 310	0. 317	0. 292
Kidney	0. 236	0. 232	0. 232	0. 172
Rectosigmoid	0. 224	0. 232	0. 236	0. 187
Lymphosarcoma	0. 206	0. 206	0. 191	0. 135
Vulva	0. 199	0. 206	0. 204	0. 208
Hodgkin's disease	0. 173	0. 169	0. 117	0. 070
Thyroid gland	0. 166	0. 166	0. 124	0. 083
Brain	0. 163	0. 144	0. 108	0. 023
Esophagus	0. 146	0. 151	0. 157	0. 161
Salivary gland	0. 113	0. 114	0. 090	0. 062
Bone	0. 093	0. 080	0. 069	0. 054
Mouth	0. 080	0. 083	0. 083	0. 074
Tongue	0. 070	0. 073	0. 074	0. 061
Lip	0. 040	0. 041	0. 040	0. 034
Larynx	0. 034	0. 035	0. 034	0. 025
Pharynx	0. 032	0. 032	0. 030	0. 021
All other sites	2. 399	2. 429	2. 366	1. 898
All sites	24. 990	25. 609	24. 396	18. 952

It is important to emphasize that the probabilities described in this report were derived by subjecting hypothetical life-table populations to fixed sets of age-specific cancer-incidence rates and mortality rates from all causes. It is clear that the further into the future the probabilities are projected, the less reliable they become. The probability of developing cancer, as described herein, should be interpreted chiefly as an index of the risk of developing the disease under current conditions of mortality and cancer incidence.

Tables 6 and 7 provide the data necessary for the calculation of the probability of developing cancer between any two ages specified in the tables, for each of the leading sites. This probability is obtained by summing the expected cancer cases between the two ages and dividing that total by survivors *without* cancer (l'_x) at the younger of the two ages. Thus, from table 6, the probability of developing lung cancer among males between 30 and 60 years of age is

$$\frac{4.22+17.61+\ldots+344.41}{93,956}=\frac{761.82}{93,956}=0.0081.$$

The *total* survivors to age x (l_x) is included in these tables for purposes of comparison with the population surviving to age x *without* cancer (l'_x). It is to be noted, however, that the survivors *without* cancer (l'_x) are to be used in calculating the desired probabilities.

The level of cancer probabilities presented in this report is somewhat lower than those reported by Cutler and Haenszel (*17*). The results reported by Cutler and Haenszel were based on surveys conducted by the National Cancer Institute in 1937–39 and 1947–48 in 10 urban areas. The lower probabilities in this report cannot, to any appreciable degree, be attributed to the fact that second independent primary neoplasms were excluded from the incidence of new cases. This factor was taken into account in the methodology by basing the probabilities on the "cancer-free" population. However, as was noted earlier, consideration could not be given to diagnosed cases never reported (while alive or by death certificate) to the New York State Health Department; and to this extent the probabilities reported herein understate the actual levels. On the other hand, the higher probabilities reported by Cutler and Haenszel may be due, at least in part, to two additional factors. First, the incidence rates obtained by a survey (as distinct from a continuous mandatory reporting system such as that in upstate New York) may be inflated by inclusion of old cases (that is, some cases actually discovered prior to the period covered by the survey are likely to be counted as new cases during the period of the survey). Secondly, the surveys conducted by the National Cancer Institute were performed in urban areas and, as stated by the authors in their report, ". . . current evidence suggests that [incidence] rates are higher in urban than in rural areas."

TABLE 6.—*Expected cancer cases in life-table population, by age, for 12 leading male sites used in determining probability of developing cancer, New York State, exclusive of New York City, 1949–51*

Age x to $x+n$	Survivors to exact age x		Number of cases						
	Total* (l_x)	Without cancer (l'_x)	All sites	Skin	Prostate	Lung and bronchus	Stomach	Large intestine	Small intestine
0–5	100, 000	100, 000	82. 55	0. 92	—	—	—	—	—
5–10	96, 732	96, 719	44. 46	1. 11	—	—	—	—	—
10–15	96, 413	96, 392	39. 66	0. 67	—	—	—	—	—
15–20	96, 101	96, 071	70. 92	2. 20	—	—	—	0. 72	—
20–25	95, 545	95, 492	90. 81	7. 52	—	1. 38	0. 67	1. 38	0. 67
25–30	94, 812	94, 726	139. 01	23. 57	—	1. 84	1. 23	4. 82	0. 61
30–35	94, 109	93, 956	206. 64	33. 13	—	4. 22	5. 44	9. 04	0. 61
35–40	93, 265	93, 015	326. 72	64. 35	—	17. 61	22. 47	23. 67	—
40–45	91, 954	91, 565	504. 18	106. 85	3. 14	46. 87	32. 49	34. 36	1. 87
45–50	89, 902	89, 304	877. 80	163. 89	10. 60	118. 14	65. 67	64. 35	2. 65
50–55	86, 493	85, 575	1, 460. 44	245. 07	30. 40	230. 57	114. 95	110. 95	1. 98
55–60	81, 334	79, 919	2, 164. 05	279. 77	103. 13	344. 41	174. 62	186. 28	2. 06
60–65	73, 743	71, 728	2, 933. 77	374. 56	242. 51	432. 29	256. 94	254. 05	8. 65
65–70	63, 627	60, 916	3, 320. 05	403. 95	374. 78	362. 94	363. 72	302. 19	11. 84
70–75	51, 246	47, 976	3, 450. 44	431. 86	545. 77	306. 46	385. 06	309. 98	4. 42
75–80	37, 098	33, 643	2, 757. 83	381. 78	575. 95	152. 89	270. 15	256. 09	5. 63
80–85	23, 291	20, 250	1, 907. 40	248. 74	447. 96	67. 14	201. 41	189. 31	1. 10
85+	11, 194	9, 116	1, 195. 50	218. 60	279. 12	27. 17	98. 80	103. 74	1. 23

TABLE 6.—(Continued)

Age x to $x+n$	Survivors to exact age x		Number of cases						
	Total* (l_x)	Without cancer (l'_x)	Rectum	Bladder	Leukemia and aleukemia	Pancreas	Lip	Esophagus	Larynx
0–5	100, 000	100, 000	1. 36	2. 28	28. 55	—	0. 44	—	—
5–10	96, 732	96, 719	—	—	17. 43	—	—	—	—
10–15	96, 413	96, 392	—	—	13. 24	0. 67	—	—	—
15–20	96, 101	96, 071	—	—	17. 01	—	—	—	—
20–25	95, 545	95, 492	0. 67	2. 05	7. 52	0. 67	1. 38	—	—
25–30	94, 812	94, 726	3. 64	1. 79	11. 48	—	0. 61	—	—
30–35	94, 109	93, 956	7. 83	4. 83	23. 48	1. 83	6. 04	—	1. 22
35–40	93, 265	93, 015	9. 73	6. 67	11. 54	4. 86	8. 52	1. 81	3. 06
40–45	91, 954	91, 565	26. 26	13. 74	18. 11	11. 24	14. 97	3. 14	8. 10
45–50	89, 902	89, 304	44. 47	34. 49	28. 53	16. 61	26. 54	11. 92	21. 24
50–55	86, 493	85, 575	85. 22	58. 78	39. 65	50. 20	31. 03	22. 45	39. 65
55–60	81, 334	79, 919	114. 79	107. 25	49. 49	66. 01	50. 18	51. 55	62. 55
60–65	73, 743	71, 728	171. 76	150. 82	67. 13	88. 76	61. 34	83. 73	62. 78
65–70	63, 627	60, 916	209. 08	190. 93	89. 95	95. 48	75. 75	89. 95	74. 17
70–75	51, 246	47, 976	204. 00	197. 83	83. 02	112. 15	72. 41	89. 19	52. 10
75–80	37, 098	33, 643	189. 49	147. 28	61. 90	70. 35	61. 90	60. 04	38. 46
80–85	23, 291	20, 250	86. 95	118. 87	35. 22	56. 13	42. 93	44. 03	24. 21
85+	11, 194	9, 116	69. 16	102. 51	18. 53	43. 23	32. 11	18. 53	14. 82

*From 1949-51 life table for New York State, exclusive of New York City.

TABLE 7.—*Expected cancer cases in life-table population, by age, for 12 leading female sites used in determining probability of developing cancer, New York State, exclusive of New York City, 1949–51*

Age x to $x+n$	Survivors to exact age x		Number of cases						
	Total* (l_x)	Without cancer (l'_x)	All sites	Breast	Large intestine	Small intestine	Skin	Cervix	Fundus uteri
0–5	100, 000	100, 000	70. 53	—	—	—	1. 42	—	—
5–10	97, 444	97, 428	48. 51	—	1. 17	—	1. 17	—	0. 58
10–15	97, 220	97, 195	37. 88	0. 73	1. 51	—	3. 69	—	—
15–20	97, 050	97, 008	70. 94	0. 73	4. 41	—	8. 04	—	0. 73
20–25	96, 769	96, 694	111. 67	5. 07	3. 82	—	17. 15	10. 77	5. 07
25–30	96, 417	96, 274	198. 46	25. 69	9. 14	0. 58	17. 12	41. 08	8. 56
30–35	95, 960	95, 700	361. 47	74. 01	17. 37	—	42. 39	104. 10	9. 85
35–40	95, 352	94, 851	660. 55	182. 03	39. 72	1. 19	58. 13	173. 73	29. 04
40–45	94, 394	93, 520	1, 114. 73	362. 28	66. 41	—	77. 14	240. 29	55. 02
45–50	92, 964	91, 470	1, 533. 03	444. 82	99. 63	2. 07	123. 15	269. 10	134. 23
50–55	90, 694	88, 499	1, 993. 25	483. 51	184. 64	0. 71	177. 64	260. 57	190. 88
55–60	87, 448	84, 402	2, 433. 59	576. 70	234. 72	3. 75	190. 49	264. 72	255. 73
60–65	82, 688	78, 814	2, 930. 05	639. 37	290. 53	5. 75	274. 14	245. 42	247. 88
65–70	75, 584	70, 838	3, 190. 69	668. 65	351. 09	6. 17	310. 50	238. 19	216. 12
70–75	65, 802	60, 469	3, 463. 36	727. 44	415. 96	7. 02	385. 83	172. 82	187. 88
75–80	52, 211	46, 752	2, 960. 65	608. 52	398. 77	6. 56	316. 83	149. 68	138. 75
80–85	36, 193	31, 486	2, 196. 44	425. 94	324. 45	2. 67	260. 37	81. 44	76. 11
85+	19, 557	16, 310	1, 614. 06	318. 33	206. 24	4. 48	240. 62	67. 26	43. 34

TABLE 7.—(Continued)

Age x to $x+n$	Survivors to exact age x		Number of cases						
	Total* (l_x)	Without cancer (l'_x)	Stomach	Ovary	Rectum	Pancreas	Bladder	Leukemia and aleukemia	Lung and bronchus
0–5	100, 000	100, 000	—	—	0. 98	—	—	24. 29	0. 49
5–10	97, 444	97, 428	—	—	—	—	—	21. 31	—
10–15	97, 220	97, 195	—	0. 73	0. 73	—	0. 73	10. 39	—
15–20	97, 050	97, 008	0. 73	2. 18	0. 73	—	—	10. 95	—
20–25	96, 769	96, 694	—	5. 70	0. 63	0. 63	—	10. 77	0. 63
25–30	96, 417	96, 274	0. 58	10. 25	3. 42	—	0. 58	11. 40	—
30–35	95, 960	95, 700	4. 07	15. 02	3. 49	1. 72	0. 57	11. 00	6. 31
35–40	95, 352	94, 851	11. 25	33. 22	17. 80	2. 37	3. 56	11. 86	3. 51
40–45	94, 394	93, 520	20. 25	61. 96	24. 65	5. 67	1. 87	18. 32	6. 28
45–50	92, 964	91, 470	22. 84	84. 42	37. 36	22. 84	9. 01	26. 97	15. 85
50–55	90, 694	88, 499	61. 31	123. 33	68. 26	24. 39	18. 82	23. 68	28. 58
55–60	87, 448	84, 402	76. 49	133. 51	81. 73	38. 27	32. 26	33. 75	35. 97
60–65	82, 688	78, 814	140. 36	131. 32	120. 65	64. 83	52. 53	47. 61	41. 02
65–70	75, 584	70, 838	182. 59	131. 42	127. 91	89. 98	54. 68	58. 23	44. 14
70–75	65, 802	60, 469	228. 09	127. 60	179. 84	69. 34	113. 52	70. 32	67. 36
75–80	52, 211	46, 752	248. 00	72. 10	135. 47	88. 48	102. 69	52. 44	54. 64
80–85	36, 193	31, 486	209. 63	33. 38	124. 18	73. 44	77. 44	42. 72	36. 05
85+	19, 557	16, 310	141. 98	26. 90	88. 18	47. 83	59. 78	22. 42	28. 39

*From 1949–51 life table for New York State, exclusive of New York City.

Summary

In order to supply information regarding the probability of developing one or another form of cancer, a study was undertaken utilizing the cancer-morbidity data for New York State (exclusive of New York City) covering the two periods 1942–44 and 1949–51. Life-table technique was employed to obtain this information, and the details of the methodology were explained at some length. A summary of the findings was presented in several tables and some of the significant results were further discussed.

For the great majority of the more than 25 sites studied, among males and females alike, there has been an increase in the probability of developing cancer. For some sites, such as lung cancer among males, the rising risk is relatively great, particularly in view of the short study span of 7 years. The over-all picture can perhaps best be summarized by the following findings:

1) The probability of developing cancer is greater among females than males, but the gap is narrowing.
2) The relative cancer risk to males has increased by 38 percent from 1942–44 to 1949–51.
3) The relative cancer risk to females has increased by 28 percent from 1942–44 to 1949–51.
4) According to present experience, 1 in 4 females and more than 1 of every 5 males may be expected to develop some form of the disease during their lifetime.
5) More than 50 percent of the total cancer risk among males is due to malignancies of the skin, prostate, lung, stomach, and intestines.
6) The probability of developing lung cancer among males has more than doubled over the study span of 7 years.
7) More than 50 percent of the total cancer risk among females is confined to cancers of the breast, intestines, skin, and cervix uteri.
8) Cancer of the breast alone accounts for more than one fifth of the total cancer risk among females.
9) The change in the probability of developing cancer over a period of time is not due solely to the force of cancer morbidity, but is considerably influenced by changes in non-cancer mortality. About 40 percent of the observed increase in the probability of eventually developing cancer among males, and approximately two thirds of the observed increase among females, was due to the general decline from 1939–41 to 1949–51 in non-cancer mortality.

The findings clearly point to the enormity of the cancer problem and to its rapidly increasing hazard.

References

(1) GRISWOLD, M. H.: Fifteen years' experience with the Connecticut cancer register. Connecticut Health Bull. 65: 2 1951.

(2) LEVIN, M. L.: Problems in human cancer statistics. Read before Sloan-Kettering Institute Seminar, Memorial Hospital, New York City, December 8, 1948.

(3) GERHARDT, P. R., GOLDBERG, I. D., and LEVIN, M. L.: Incidence, mortality, and treatment of breast cancer in New York State. New York J. Med. 55: 2945–2951, 1955.

(4) LEVIN, M. L., and GOLDSTEIN, H.: Cancer incidence, mortality and expectancy in upstate New York. Presented at the Fourth Internat. Cancer Res. Congr., St. Louis, Mo., September 2–7, 1947.

(5) LEVIN, M. L.: Cancer reporting in New York State. New York J. Med., 44: 880–883, 1944.

(6) WARREN, S., and GATES, O.: Multiple primary malignant tumors. A survey of the literature and a statistical study. Am. J. Cancer 16: 1358–1414, 1932.

(7) ———: Cancer of the skin in relation to multiple malignant growths. J. A. M. A. 115: 1705–1707, 1940.

(8) WARREN, S., and EHRENREICH, T.: Multiple primary malignant tumors and susceptibility to cancer. Cancer Res. 4: 554–570, 1944.

(9) PELLER, S.: Metachronous multiple malignancies in 5,876 cancer patients. Am. J. Hyg. 34: 1–11, 1941.

(10) ———: Race, stock and environment in human cancer. J. Hered. 35: 175–181, 1944.

(11) LOMBARD, H. L., and WARREN, S.: Association of other malignant tumors with cancer of the skin. Am. J. Pub. Health 33: 533–536, 1943.

(12) LOMBARD, H. L., LEVIN, M. L., and WARREN, S.: Multiple malignant growths. Cancer Res. 6: 436–440, 1946.

(13) MIDER, G. B., SCHILLING, J. A., DONOVAN, J. C., and RENDALL, E. S.: Multiple cancer; study of other cancers arising in patients with primary malignant neoplasms of stomach, uterus, breast, large intestine, or hematopoietic system. Cancer 5: 1104–1109, 1952.

(14) REED, L., and MERRELL, M.: A short method for constructing an abridged life table. Am. J. Hyg. 30: 33–62 (Sept.), 1939. Reprinted as Vital Statistics—Special Reports 9: 681–712, 1940.

(15) DUBLIN, L. I., LOTKA, A. J., and SPIEGELMAN, M.: Length of Life. New York, The Ronald Press Co., 1949.

(16) BUREAU OF CANCER CONTROL: Annual Report. Albany, New York State Department of Health, 1953.

(17) CUTLER, S. J., and HAENSZEL, W. M.: The magnitude of the cancer problem. Pub. Health Rep. 69: 333–339, 1954.

Digital Computer Studies of Cell Multiplication by Monte Carlo Methods [1]

JOSEPH G. HOFFMAN, *Biophysics Division, Roswell Park Memorial Institute, Buffalo, New York, and* NICHOLAS METROPOLIS *and* VERNA GARDINER, *Theoretical Division, Los Alamos Scientific Laboratory,*[2] *Los Alamos, New Mexico*

This report describes studies of cell multiplication where randomness exists in the time parameters of the life cycle of the cell. Randomness in the incidence of mitosis and in the duration of division are amenable to analysis by the Monte Carlo method (*1*). The digital computer (the Maniac located at the Los Alamos Scientific Laboratories) was employed since it gives results which are readily comparable with experimental findings. The purpose of the calculations was to examine the properties of the growth of a clone, or colony, stemming from a single cell. The growth rate, incidence of mitosis, and age structure in populations were studied in relation to the time parameters of a single cell. The deterministic model in which the population number, n, increases as $n=e^{\beta t}$, where β is the exponential constant and t the time elapsed, gives little insight into the properties of cell populations encountered experimentally.

Stochastic models of cell multiplication were therefore set up to provide a theoretical basis for the interpretation of observed growth processes (*2*) and of possible mutations in transplantable mouse tumor cells (*3*). This was prompted also by the fact that the mathematical analysis of cell-population growth in which a cell has a rest period, or period in which it does not divide, is not yet fully developed. For further discussion of the theory the reader is referred to Kendall (*4*) and Armitage (*5*).

Methods

A free birth process is assumed in which the population increases exponentially with time. No cells are removed by death or other mechanism. The parameters are: intermitotic time, P; the duration of mitosis, T; and the cell-number doubling time, $L=T+P$. The parameters P and T are not uniquely defined except as average values. It is assumed that there are distributions for the intermitotic time and for the duration of mitosis. These are determined by the nature of the cell cycle and will be described below. It is possible that the randomness of these parameters

[1] Received for publication March 30, 1956.
[2] This work was supported by the United States Atomic Energy Commission,

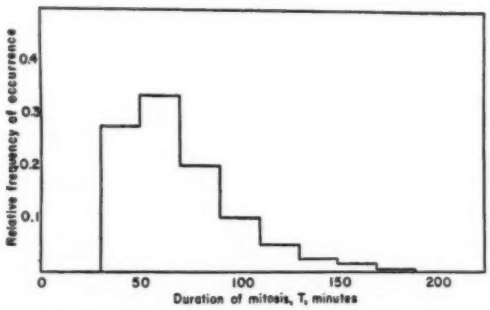
arises from molecular events which accompany cell reduplication, or it may arise from external factors.

Given the probability distributions for the parameters T and P, the digital computer carries out the sequence of cell multiplication as a series of chance events. The picture is that at time, $t=0$, a single, newly formed cell starts to grow. The computer plays the game of chance which determines the time of division, the rules of the game being carried out according to the assigned probability distribution. At the instant when cell division starts, another probability distribution takes over in another game of chance by which the computer determines the duration of mitosis.

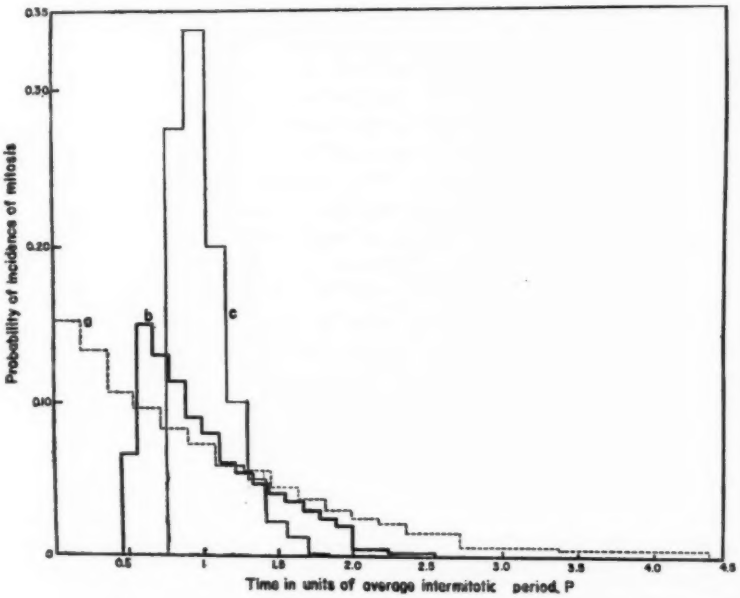
Upon completion of division the computer has 2 cells, 1 of which it puts into a storage compartment for future reference. The other cell is carried repeatedly through the mitotic cycle for the length of time for which the multiplication process is to be studied, each new daughter cell being put into a memory store for future reference. After the growth interval is ended, the computer is instructed to go back and follow through the repeated mitotic cycle with each daughter cell. In each case the new daughter cells are stored away as they are formed. Ultimately all the daughter cells have been carried through the randomly occurring growth cycles up to the end of the assigned growth period.

By means of appropriate instructions the computer prints out the number of cells in the population at prescribed times and, of these, the number which are in the process of division. At the end of the growth time, the computer prints out the number of cells of different intermitotic ages, a datum which determines the age structure, or distribution of ages in the final population. Another datum available is the distribution of lengths of time cells have been in the dividing stage. This distribution may be correlated with the observed phases of mitosis.

In the studies reported here a fixed distribution (text-fig. 1) for the duration of mitosis, T, was adopted. Since the parameter T is relatively small, usually at most a few percent of P, it has a minor effect on the multiplication process. For the incidence of intermitotic time, 3 different



TEXT-FIGURE 1.—Probability distribution for the duration time, T, of mitosis. Mean value of T is 67.8 minutes.



TEXT-FIGURE 2.—Normalized probability distribution for the intermitotic time, P. a=Yule process with negative exponential tail. b=Modified Yule process with 17-hour rest period before division can occur. c=Approximate distribution based on mouse spleen cells *in vitro* and bacterial data.

distributions, as shown in text-figure 2, were selected. They are plotted in units of average intermitotic time, L, the average being the arithmetic average determined by the product-sum method. The average time parameters for the 3 cases are given in the first 3 columns of table 1.

The magnitudes of L and T were determined by the average values reported in the literature for transplantable mouse tumors. Adenocarcinoma drbB cells inoculated subcutaneously in strain DBA mice show an average L value of 23 hours (*6*); when grown in chick embryo $L=17$ hours (*2*). The Krebs-2 ascites tumor has an L value of 16 hours (*7*). For the Marsh-Simpson tumor, another mammary adenocarcinoma,

TABLE 1.—*Average parameters in life cycles of cell models**

Case	Average duration of mitosis, T (*hours*)	Average intermitotic time, P (*hours*)	Average generation time, L (*hours*)	Calculated mitotic index (mi)†	Machine mitotic index‡
a	1. 145	21. 95	23. 10	0. 0343	0. 0456
b	1. 145	35. 84	37. 00	0. 0215	0. 0214
c	1. 145	22. 17	23. 31	0. 0340	0. 0346

*Arithmetic averages determined by product-sum method.
†Calculated theoretically from (mi)=0.693T/L.
‡Poisson average as found by Monte Carlo calculation on the digital computer.

inoculated subcutaneously in the strain MA mouse, L=46 hours (*8,9*). The distributions shown in text-figure 2 are hypothetical. Measurements of the distribution of P have been reported on bacteria by Kelly and Rahn (*10*) and Powell (*11*). The first distribution, graph a, text-figure 2, is that for the Yule process, in which the newly formed cells have a finite probability of entering division as soon as they are formed. Its average intermitotic time is 21.95 hours. With increasing time the probability diminishes approximately as a negative exponential. It will be recognized that this process is analogous to that of radioactive decay.

The Yule-process type of multiplication is included for its historic interest in the study of population growth. It was first formulated by Yule (*12*) for the analysis of mutations of species and is discussed in other applications by Feller (*13*) and by Iversen and Arley (*14*). The extent to which it is applicable to tissue cell growth is uncertain in that it presents a logical difficulty; namely, some cells may divide themselves into submicroscopic bits.

To avoid this difficulty a second distribution was made in our calculations. The Yule process was modified by the introduction of a rest period of 17 hours before division could be entered into. After that initial period the probability of dividing falls off approximately as a negative exponential as shown in graph b, text-figure 2. Its average intermitotic time is 35.84 hours.

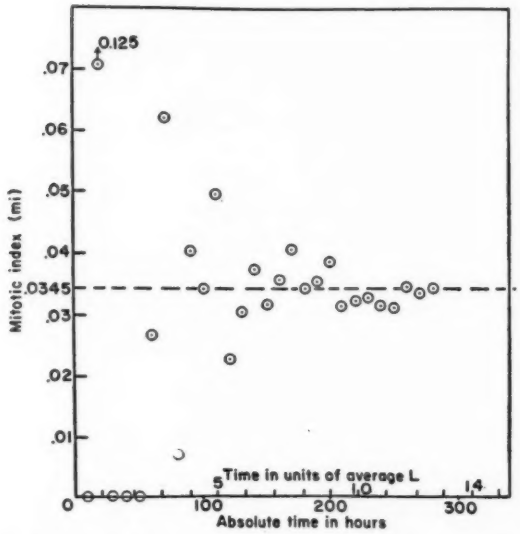
The third distribution, having an average intermitotic time of 22.17 hours, graph c, text-figure 2, was designed to have a narrower spread in P than in the first 2 cases. It ranges from 0.74 to 1.78, with the average at 1.0. This case is based upon the spread of intermitotic times suggested by the generation times measured in *Aerobacter aerogenes* by Kelly and Rahn (*10*) and also by the intermitotic times directly timed by Fell and Hughes (*15*) in mouse spleen cells *in vitro*.

Results and Discussion

Stabilization of the Population

It was found that, starting from a single cell, the time for the population to reach a steady state of growth was about 9 average generation times, or $9L$. This was the period during which major fluctuations in cell number and in the ratio of mitotic to total cell numbers subsided. A representative illustration of the fluctuations in the mitotic index of a clone is shown in text-figure 3 for case c of text-figure 2. The mitotic index is defined as the ratio of the number of mitotic cells to the sum of the mitotic plus intermitotic numbers. Case c, with its relatively narrow spread in P, might have been expected to show the most rapid approach to a steady state in its multiplication. The time of about $9L$ was also found to be required for stabilization in the other 2 cases—a and b, text-figure 2. Data similar to that of text-figure 3 are given in table 2 for case b.

Fluctuations in population size are shown in text-figure 4, where 6 selected cells in case a are followed for an interval of at least 10 average



TEXT-FIGURE 3.—Mitotic index of entire cell population having intermitotic times of graph c, text-figure 2. Fluctuations are wide until about 9 average generation times, L, have elapsed. One point, at 0.125, is off scale.

generation times. The growth curves of text-figure 4 are also representative of cases b and c except that case c has a smaller variation than the other cases. The single cells ultimately grow into populations, each having the same exponential growth constant. The important feature is that they become stabilized at different population sizes. This is the result of variable generation times in the early multiplications. Extrapolation of the growth curves back to the time axis shows that they have starting points other than a single cell at $t=0$. Some populations extrapolate back to a negative intercept on the time axis. Others intercept the time axis at positive values, which has the effect of imparting an

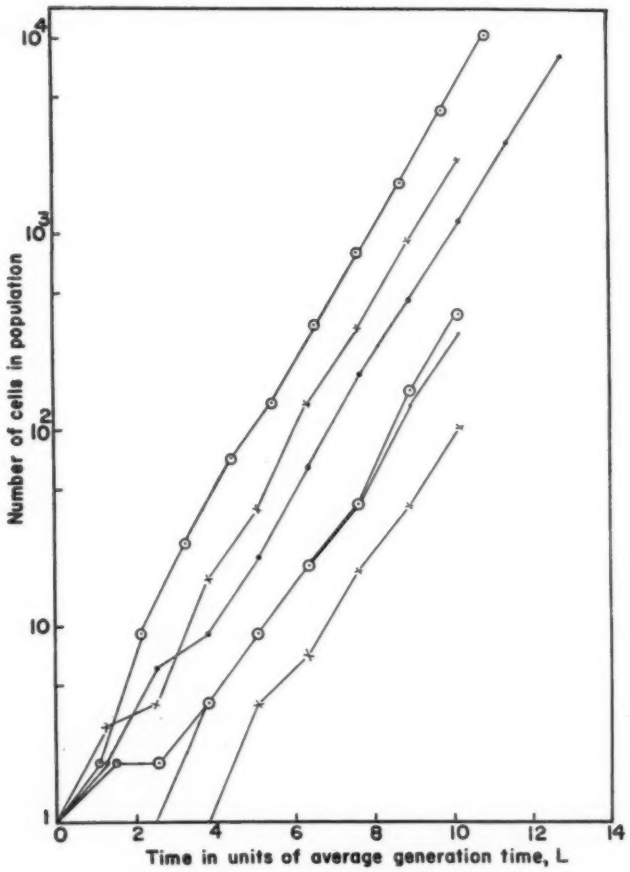
TABLE 2.—*Case b: ratio of number of mitotic to intermitotic cells*

Time* (L)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7
9. 92	. 019	. 013	. 019	. 025	. 013	. 012	. 010
10. 54	. 017	. 012	. 012	. 018	. 012	. 013	. 015
11. 16	. 024	. 015	. 020	. 019	. 023	. 009	. 018
11. 77	. 018	. 011	. 021	. 022	. 021	. 017	. 011
12. 40	. 022	. 009	. 016	. 023	. 019	. 018	. 011
13. 02	. 021	. 012	. 020	. 021	. 019	. 015	. 015
13. 64	. 021	. 013	. 026	. 020	. 019	. 013	. 014
12. 26	. 022	. 014	. 024	. 023	. 018	. 015	. 014
14. 88	. 022	. 014	. 020	. 023	. 019	. 015	. 014
Total cells†	28, 693	26, 015	6, 353	22, 038	32, 206	41, 456	12, 301

*Time in units of average generation time.
†Total cells generated from a single cell in the time 14.88L.

apparent latent period to the growth of the population. For case a, text-figure 4, latent periods as long as $3.9L$ were found. The maximum possible length may be $4.4L$ (text-fig. 2). In case b it was found to be as much as 3.2 average generation times, or $3.2L$. This latent period is a stochastic effect and is a consequence of the randomness assumed in the growth process.

It is necessary to point out the distinction between this latency and that found in the growth of large populations. Quantitative measurements on bacteria, ascites-tumor cells, and regenerating liver cells show 3 main stages in population growth. At first there is a latent period of little or no cell multiplication followed by the characteristic exponential growth. After reaching a large size the population grows slowly again, or in some cases it may decrease in size. For each type there are diverse



TEXT-FIGURE 4.—Growth of 6 different cell populations, beginning with a single cell, having intermitotic times distributed according to graph a, text-figure 2. After a time $9L$ the populations asymptotically approach the expected growth as 2^L.

causes for the duration of each of these 3 phases which need not be detailed here. The Monte Carlo study reported here pertains to the steady state of a pure birth process with exponential multiplication.

Therefore it is noteworthy that the steady state of exponential growth can show an effect resembling a lag phase. The latent period in the growth curves of single cells, text-figure 4, has no relation to the lag phase observed experimentally with large populations of bacteria and of cells. In the computer results it is seen that when the population reaches about 1,000 cells the fluctuations in numbers become relatively small. The curves show that, starting with small numbers of cells ranging from 1 to 10, a population may undergo fluctuations in size and have an apparent latent period, these properties being more marked the smaller the initial cell number.

Since mutations appear in single cells the latent period due to the stochastic effect would be manifested as a delay similar to the phenotypic delay observed in bacteria. If the spread in generation times is narrow as in case c, the delay could be insignificant compared with the total growth time of the culture. On the other hand, the data of Kelly and Rahn (*10*) for *Aerobacter aerogenes* show examples in which the spread about the average generation time is at least a factor of 4. If these ranges of generation times occur in mutants, the stochastic effect could become a part of the delay in appearance of mutant clones. Such considerations become significant also in the mutations of mouse tumor cells grown in incubating eggs.

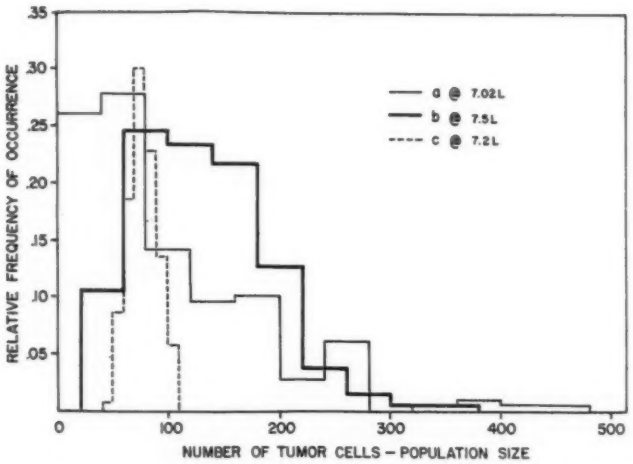
Mirand and Hoffman (*3*) have shown that a DBA mouse mammary adenocarcinoma passed from the mouse into the chick embryo and then into previously resistant mouse strains, was able to grow in the resistant strains. In this case the tumor cell in the egg has an average generation time of 16 hours.

The total growth time in the chick embryo is 11 days. Therefore a possible delay of 4 average generation times, or 64 hours, becomes significant. Even though a cell may have a short average generation time, it will be selectively eliminated if a long stochastic delay is possible. This delay becomes more significant, of course, for mutants having a relatively long average generation time.

The stochastic effect in growth leads to a distribution of population sizes which is sensitive to the distribution of generation times. In text-figure 4 the spread of times required to generate 100 cells from a single cell is $5.2L$. The corresponding range for case b was found to be $4L$ and for case c, $3L$. The frequency distribution of population sizes for approximately equal growth periods is shown in text-figure 5. The 3 cases reflect the distribution of generation times. Thus, case c has the narrowest range, while the distribution for case a has the widest, and case b is intermediate between the 2 extremes. The sensitivity of the distribution of population sizes to the distribution of generation times suggests that single-tumor-cell inocula (*16*) might afford a means for determining the latter from the former. A comparison of text-figure 5

with text-figure 2 shows qualitatively the results of Kendall's (*4*) approximation, namely, that the variance of population sizes is proportional to the variance of the generation times.

In another direction, our results show that the digital computer provides a methodological approach to the analysis of mutations occurring in a population. Analytical methods (*17*) have been developed for assessing mutation rates, the theory being based on special distributions of generation times in both cells and their mutants. The Monte Carlo method, however, is a general means for utilizing the experimental distribution of clone sizes of mutants. This type of calculation is being studied.



TEXT-FIGURE 5.—Distributions of population sizes arrived at after approximately the same generation times, starting with a single cell. Graphs a, b, and c correspond to cases a, b, and c in text-figure 2.

Incidence of Mitosis

In our computations the rate of multiplication can be calculated from the numbers of cells in the population at known times as given by the computer. With actual tissues, however, it is not always feasible to determine the generation time by this method, especially in tissues where the cells are not separable. The incidence of mitosis was therefore studied to see if it would provide other means for measuring the rate of multiplication. For this purpose the mitotic ratio was calculated. It is defined as the ratio of the number of mitotic to intermitotic cells and can be used as a close approximation for the mitotic index. The mitotic index was defined in the preceding section. The mitotic ratio is slightly larger percentagewise than the mitotic index, by approximately the mitotic ratio expressed as percent. In the particular models studied here the difference between mitotic ratio and mitotic index is negligible.

Clones having more than 5,000 cells were studied first for the incidence of mitosis. An interesting effect is shown in table 2 for case b, which gives the mitotic ratio at the generation times shown, ranging from $9.92L$ to $14.88L$ for 7 clones, each generated from a single cell. The total number of cells at $14.88L$ is given at the foot of each column. Inspection shows that the ratio in each clone seems to level off to a constant value different for each clone. The ratios range from .014 in run 2 to .023 in run 4. The semilog plot of cells numbers in each run has a common slope corresponding to a common average generation time of 37 hours. The 7 runs are presented as they were run off in succession—they were not selected from a group of runs

The ratio in table 2 may be taken as a measure of proliferation rate since it is the fractional increment of cell numbers generated in the average mitotic time, T. Yet the differences in ratio among the 7 runs show that this ratio is misleading as a measure of multiplication rate since the cells are known to have a common average generation time of 37 hours. Of fundamental interest is the persistence of the effect in large populations. Run 6 has 41,456 cells in the final clone, the largest of the 7 runs; yet its last 4 values of mitotic ratio are low. If all 7 runs are combined, the total of intermitotics is 169,062 and the total of mitotics is 3,088. The ratio is .0182, which is lower than the mitotic index determined as a Poisson average (described below and given in table 1, column 5).

An explanation of the effect was found in the fluctuations of the relative increment of cells—in other words the exponential constant. It was found that the relative increment oscillated about the average value and that the values of mitotic ratio shifted accordingly as the times of recording were changed. The oscillations are not regularly sustained nor do they have a fixed period. They suggest that there is a probability aftereffect *(18)*. Such an effect might be reflected in the relative frequency of the phases of the mitotic process. This type of fluctuation also has a bearing on the interpretation of the frequency of mitoses seen in large populations of cells—for example, when a section or smear is scanned.

The mitotic ratio is not always a readily measurable property of a large clone of cells. On the other hand, the incidence of mitoses in groups of 100 cells more closely approximates practical histologic methods. Therefore we had the computer generate groups of 100 cells, starting from a single cell, in order to ascertain the Poisson average.

A special sequence of counting had the computer stop cell multiplication at the instant when the 99th and 100th cells were formed in the fission of the 99th. At this instant the 99th and 100th cells had a negligible probability of being in mitosis and were therefore excluded from the count of mitoses among the remaining 98 cells. The method thus determined the incidence of mitoses among clones of 98 cells. In all 3 cases, over 400 clones were computed and the mitotic index was determined. The incidence of mitoses in the clones followed the Poisson distribution. Its average was then compared with the value computed from the average time parameters.

The mitotic index (mi) for pure exponential growth can be calculated deterministically from the parameters in the life cycle of the cells (*19*). If the characteristic exponential constant for the growth of the populations is β, then the mitotic index is: $(\text{mi})=\beta T$, where T is the duration of mitosis. This also can be put into the form: $(\text{mi})=0.693T/L$, where T and L are average values determined by the product-sum method over the probability distributions for T and L. Table 1 shows the theoretical values of the mitotic index thus calculated and, for comparison, the Poisson averages from the 100-cell method on the digital computer. In cases b and c, the agreement is good. In case a, however, it was found that the cell populations had a generation time of 17.9 hours rather than the 23.1-hour average generation time determined by the product-sum method. This discrepancy was again found in a second example of the Yule process (like case a)—the generation time by the product-sum method should have been 20.03 hours, whereas the populations produced on the machine had a generation time of $L=16.60$ hours. The mathematical analysis of the discrepancy, which also appears in the mitotic index, will be described in another paper. The product-sum generation time was found to be the same as the computer generation time in cases b and c.

The Poisson distribution is the experimentally reported distribution for mitoses in mouse ear epidermis (*20*), in adenocarcinoma dbrB (*21*), in the Marsh-Simpson tumor (*22*), and in regenerating rat liver (*23*). The runs by the machine for 100 cells per clone are comparable with the mouse-epidermis data. As for tissues such as tumor and liver, three-dimensional extension requires that thin sections be made for counting of cells and their mitoses. Such slices probably contain groups of 100 cells in which the cells are more than 7 generations removed from the common ancestor. The assumption has to be made that the population groups counted in a section are comparable to those arising from a single cell and are thus comparable with the calculated clones.

The Poisson distribution of mitoses sets a stringent criterion for uniformity of growth of a cell population. Its appearance in a given tissue means that every cell has the same probability of being found in mitosis, which in turn implies that the physiologic factors controlling growth are uniform. The observed Poisson incidence in tissues also implies variability in the generation time of each cell. This is to be contrasted with a hypothetical tissue in which cells have a single, fixed generation time, L. They then have to have the phases of their division cycles randomly distributed relative to one another in order to show a Poisson incidence of mitosis. Such a phase randomness might arise from the random availability of metabolites. This illustrates the possible influence of the cells' external environment as compared with intracellular mechanisms as a source of randomness in the life cycle. A fixed value of L can be detected in the sizes of clones from single cells—the clones will have cell numbers corresponding only to integral powers of 2. When L varies, clones having 3, 5, 6, 7, 9, etc. cells will be seen, their duration time depending on the range of L.

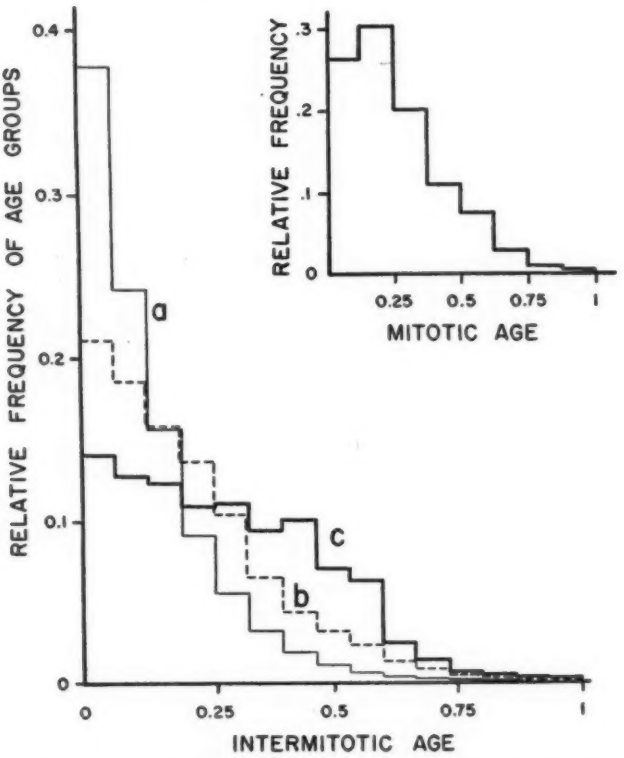
Distribution of Cell Ages

The role of the distribution of intermitotic time in determining the distribution of ages of cells in the population is shown in text-figure 6, where graphs a, b, and c correspond to the distributions a, b, and c in text-figure 2. Intermitotic age is the time lapsed since a cell was formed as a result of a binary fission. The inset in text-figure 6 shows the mitotic-age distribution, which reflects the distribution of mitotic times in text-figure 1. The mitotic age is the length of time a cell has been in the process of dividing. The intermitotic age structure for the Yule process, case a, is a pure negative exponential. Most cells are young; 88 percent of all cells have ages less than 30 percent of the possible maximum. In the modified Yule process, case b, the rest period introduces a flat top to the age structure in the low age range because no cells can divide for a certain period after birth. Once they attain the age at which division can begin, the population again falls off as a negative exponential. Here, too, the majority of cells in the entire population are young cells; over 60 percent have ages less than 40 percent of the possible maximum.

In case c, where cells have to wait until they have an age of about 75 percent of the average intermitotic period before dividing, the distribution is flat, with a falling off beginning at about 40 percent of the maximum possible age. The longer rest period before mitosis can begin imposes a longer flat top on the distribution. The tailing off at higher ages again follows a negative exponential decay.

With the age structure it is possible to draw inferences about the distribution of cell sizes, provided that the growth of cell volume with intermitotic age is known. In a first approximation, a mouse tumor, the volume of which has an exponential growth rate, may be expected to have its constituent cells growing at that same rate. The distribution of cell volumes of the dbrB tumor (*21*) and the Marsh-Simpson tumor (*22*) have been examined on the assumption that each cell grows with the same exponential constant as does the whole tumor mass. Assuming age distributions similar to those given in case b above, a Monte Carlo calculation, using tables of random numbers instead of the digital computer, was carried out to assess the shape of the distribution of cell volumes as measured experimentally (*21*). It was in the calculation of these data that the incompatibility of the Yule process in cell growth became most apparent. It was not possible to assign a lower limit of cell size as is found experimentally.

The resulting distribution of generation times in the dbrB tumor is of the form of case b, with an average generation time of 23 hours. This distribution, analyzed according to the method of Kendall (*4*) and Finney and Martin (*24*), fits the case in which 18 randomly occurring serial events in the cell determine the onset of division. These authors analyzed the data of Kelly and Rahn (*10*) on *Aerobacter aerogenes*; Kendall estimated 20 and Finney and Martin estimated 26 discrete events which had to transpire in that organism before division could occur. In the dbrB tumor



TEXT-FIGURE 6.—Age distribution of cells plotted as maximum possible ages. Graphs a, b, and c give intermitotic ages of populations having distributions a, b, and c in text-figure 2. Inset: distributions of mitotic ages in all 3 cases—a, b, and c.

cell our estimate of 18 stochastic events is subject to the assumption that each tumor cell grows at the same exponential rate as does the whole tumor. It is a crude approximation justified only by the fact that the average rate of cell growth, based on large numbers of cells, is the same as that of the whole tumor. This point will have to await clarification by data on tumor-cell volume as a function of intermitotic age.

The shape of this function may also have a bearing on the interpretation given to the numbers of the stochastic events derived from the analysis. For a discussion of the possible significance of these numbers the reader is referred to Kendall (*4*), Rahn (*25*), and Powell (*11*). Rahn postulated that the genes in bacteria are synthesized concurrently in the intermitotic period. Kendall has suggested the model of a required time sequence of synthesis of cell parts. Each of these concepts leads to a characteristic distribution of generation times. They are, however, based on an assumed independence of cell growth. Since cells grow in close contact in their cultures or in tissues, it is conceivable that their generation times are influenced by the presence of their nearest neighbors.

Summary

1) The Monte Carlo method of generating cell populations is described for cells in which the intermitotic time and duration of mitosis occur randomly. Three different kinds of growth processes are studied in which the rest period between divisions and the spread of intermitotic times are varied.

2) The effect of the stochastic process on the cell populations in exponential multiplication is shown to bring about a distribution of population sizes and to introduce a latent period in their growth.

3) The incidence of mitosis in the cell populations follows the Poisson distribution in all 3 cases studied. The age distribution and the distribution of population sizes are sensitive to the distribution of intermitotic times.

4) The Monte Carlo method and the digital computer provide a methodological approach to the growth of cell populations. The results permit a ready comparison with experimental data.

Bibliography

(*1*) HOFFMAN, J. G., METROPOLIS, N., and GARDINER, V.: Study of tumor cell populations by Monte Carlo methods. Science 122: 465–466, 1955.

(*2*) MIRAND, E. A., and HOFFMAN, J. G.: Heterotransplantation and growth rate of Dba mouse tumor in chick embryo yolk-sac. Exper. Med. & Surg. 13: 183–188, 1955.

(*3*) MIRAND, E. A., and HOFFMAN, J. G.: Change of tumor specificity after serial passage on the chick chorio-allantoic membrane. Proc. Soc. Exper. Biol. & Med. 88: 656–660, 1955.

(*4*) KENDALL, D. C.: Stochastic processes and the growth of bacterial colonies. Symp. Soc. Exper. Biol. VII: 55–65, 1953.

(*5*) ARMITAGE, P.: Statistical concepts in the theory of bacterial mutation. J. Hyg. 51: 162–184, 1953.

(*6*) REINHARD, M. C., GOLTZ, H. L., WARNER, S. G., and MIRAND, E. A.: Growth rate and percentage takes following inoculation of known numbers of viable mouse tumor cells. Exper. Med. & Surg. 10: 254–258, 1952.

(*7*) PATT, H. M., and BLACKFORD, M. E.: Quantitative studies of the growth response of the Krebs ascites tumor. Cancer Res. 14: 391–396, 1954.

(*8*) HOFFMAN, J. G., GOLTZ, H. L., REINHARD, M. C., and WARNER, S. G.: Quantitative determination of the growth of a transplantable mouse adenocarcinoma. Cancer Res. 3: 237–242, 1943.

(*9*) REINHARD, M. C., GOLTZ, H. L., and WARNER, S. G.: Further studies on the quantitative determination of the growth of a transplantable mouse adenocarcinoma. Cancer Res. 5: 102–106, 1945.

(*10*) KELLY, C. D., and RAHN, O.: The growth of individual bacterial cells. J. Bact. 23: 147–153, 1932.

(*11*) POWELL, E. O.: Some features of the generation times of individual bacteria. Biometrika 42: 16–43, 1955.

(*12*) YULE, G. U.: A mathematical theory of evolution based on conclusions of Dr. J. C. Willis, F.R.S. Philos. Tr. Roy. Soc., London, s.B 213: 21–87, 1925.

(*13*) FELLER, W.: An Introduction to Probability Theory and Its Applications. New York, John Wiley & Sons, Inc., 1950.

(*14*) IVERSEN, S., and ARLEY, N.: On the mechanism of experimental carcinogenesis. Acta path. et microbiol. scandinav. 27: 773–803, 1950.

(*15*) FELL, H. B., and HUGHES, A. F.: Mitosis in the mouse: a study of living and fixed cells in tissue cultures. Quart. J. Micr. Sc. 90: 355–380, 1949.

(*16*) HAUSCHKA, T. S.: Cell population studies on mouse ascites tumors. Trans. New York Acad. Sc. (II) 16: 64–73, 1953.

(*17*) ARMITAGE, P.: The statistical theory of bacterial populations subject to mutation. J. Roy. Stat. Soc., s.B 14: 1–40, 1952.

(*18*) CHANDRASEKHAR, S.: Stochastic problems in physics and astronomy. Rev. Mod. Phys. 15: 1–89, 1943.

(*19*) HOFFMAN, J. G.: Theory of the mitotic index and its application to tissue growth measurement. Bull. Math. Biophys. 11: 139–144, 1949.

(*20*) IVERSEN, S., and EDELSTEIN, J. M.: On the mechanism of experimental carcinogenesis. 4. The early mitotic effect of 9,10-dimethyl-1,2-benzanthracene and 1,2,5,6-dibenzanthracene on epidermal cells of the mouse ear. Acta path. et microbiol. scandinav. 30: 213–222, 1952.

(*21*) HOFFMAN, J. G.: The Size and Growth of Tissue Cells. Springfield, Ill., Charles C Thomas, 1953, p. 85.

(*22*) HOFFMAN, J. G., and RAINEAR, H. P.: Cell growth in the Marsh-Simpson transplantable mouse adenocarcinoma. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 21, 1954.

(*23*) BRUES, A. M., and MARBLE, B. B.: An analysis of mitosis in liver restoration. J. Exper. Med. 65: 15–27, 1937.

(*24*) FINNEY, D. J., and MARTIN, L.: A re-examination of Rahn's data on the number of genes in bacteria. Biometrics 7: 133–144, 1951.

(*25*) RAHN, O.: A chemical explanation of the variability of the growth rate. J. Gen. Physiol. 15: 257–277, 1932.

The Metabolism of L-Histidine-2-C^{14} in the Normal and Hepatoma-Bearing Rat. II. Protein and Nucleic Acid Relationships [1]

JAMES C. REID, ODETTE S. TEMMER, *and* MARTHA O. BACON,[2] *Laboratory of Physiology, National Cancer Institute,*[3] *Bethesda, Maryland*

Histidine is a nutrilite of basic importance in the mammalian economy. In the biochemistry of cancer it is of particular interest because of its role as a precursor of both protein and nucleic acids. In this paper a tracer study of these aspects of histidine metabolism is reported.

Experimental

Adult Osborne-Mendel rats, in 2 groups of 3, were each given by stomach tube 2.33 mg. of L-histidine labeled in position 2 of the ring with carbon 14. These were the same rats [4] used in an earlier experiment (*1*). The dose was 3.7×10^7 disintegrations per minute. The rats in one group were normal; the others bore primary hepatomas induced by feeding a synthetic diet containing 0.06 percent *N,N*-dimethyl-*p*-phenylazoaniline (*p*-dimethylaminoazobenzene, DAB) for a period of 3 months. After 3 months' feeding, the rats were placed on the synthetic diet without DAB until tumors arose. The composition of the diet has been given earlier.[5]

The tumor weights were between 4 and 10 gm. Microscopic examination [6] revealed tissues predominantly parenchymal in appearance. There was no central necrosis, and focal necrosis, fibrosis, and proliferation of bile-duct cells were minimal. The material appeared to resemble very closely that described in a quantitative study by Daoust (*2*), who found the percentage of parenchymal cells in DAB hepatoma to be about the same as in normal liver.

Approximately 3 months elapsed between the cessation of DAB feeding and the experiment. The normal animals were placed on the synthetic diet 2 days before the beginning of the experiment and were maintained on this diet through the duration of the experiment. The animals were placed in individual metabolism cages with provision for the collection of urine, feces, and exhaled carbon dioxide. At intervals of 12, 24, and 72

[1] Received for publication April 5, 1956.

[2] *Present address:* National Institute of Mental Health.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] We are indebted to Dr. Julius White, National Cancer Institute, for kindly supplying the rats from his colony.

[5] The diet contained 6 percent crude casein. In our earlier communication this was erroneously reported to be 12 percent.

[6] We are indebted to Dr. Thelma B. Dunn for the pathologic examinations.

hours after administration of the histidine, 1 rat from each group was killed and dissected. In the case of the tumor-bearing animals the remaining liver (hereafter called host liver) was separated from the tumor nodules and worked up separately. The tissues were dried by lyophilization. Gross radioactivity determinations were made by burning aliquots of the tissues in a combustion train, collecting the carbon dioxide, and counting it as barium carbonate in a windowless flow counter. Isolated metabolites were assayed directly as "infinitely thin" layers on glass plates.

The individual livers and tumors were defatted and extracted with cold 5 percent trichloroacetic acid. They were then digested with alkali under the conditions of Schmidt and Thannhauser (*3*) for the extraction of nucleic acids. After digestion the solution was acidified to *p*H 4.9 with perchloric acid, which precipitated the bulk of the protein and some DNA. After filtration, the *p*H of the filtrate was lowered to 2.0 by the addition of more perchloric acid, which precipitated the bulk of the DNA. After removal of the precipitate by high-speed centrifugation the supernatant was raised to *p*H 7.0 and the ribonucleotides were precipitated as their mercuric complexes by the addition of a solution of mercuric perchlorate. After removal of mercury with hydrogen sulfide the ribonucleotides were purified by chromatography on Dowex-1 with dilute hydrochloric acid as the eluent (*4*), followed by rechromatography of each component separately on Dowex-1 with formic acid of various concentrations.

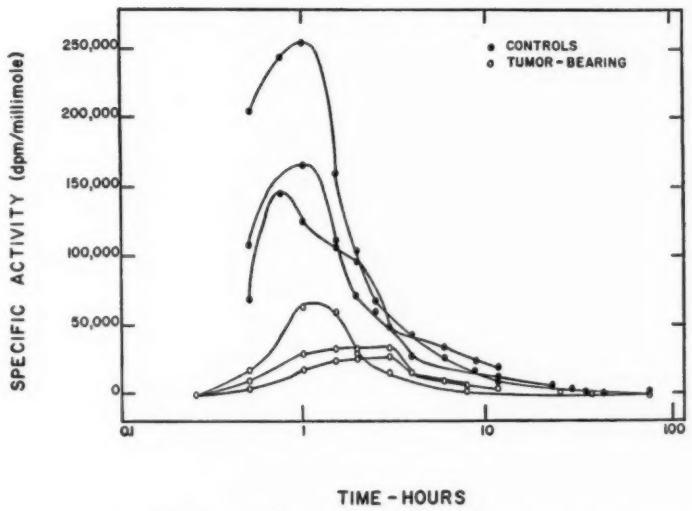
DNA purines were extracted from the crude DNA precipitate with hot trichloroacetic acid (*5*) and recovered as their mercury complexes. After removal of mercury they were chromatographed on Dowex-50 in 2 *M* hydrochloric acid and then purified further by rechromatography of each component separately on Dowex-50 with 1 *M* hydrochloric acid.

The main protein residue was hydrolyzed and all the constituent amino acids were isolated by chromatography on Dowex-50 (*6*,*7*).

Results

Carbon dioxide.—Text-figure 1 is a plot of the specific activity of the respiratory carbon dioxide against time. Time is plotted logarithmically for convenience to foreshorten the scale. It is seen that the 3 normal animals present a relatively uniform picture although there is considerable variation in the maximum specific activity. The curve exhibits a comparatively simple form with a rapid rise to a maximum at about 1 hour, followed by a rapid drop through a shoulder region and then a much slower drop beyond 12 hours.

The shape of the curves for the 12- and 24-hour tumor-bearing rats is entirely different. The initial rise is less rapid, the peak is much broader, the time necessary to reach maximum specific activity is increased, and the maximum specific activity obtained is much lower. The 72-hour cancerous animal exhibits a curve more similar in shape to the normal animals in that the flattening of the peak is not so pronounced; but a disturbance of $C^{14}O_2$ output is evident, nevertheless, in the more gradual front, the increase in time necessary to reach maximum specific activity,



TEXT-FIGURE 1.—Specific activity-time relationships of carbon dioxide output.

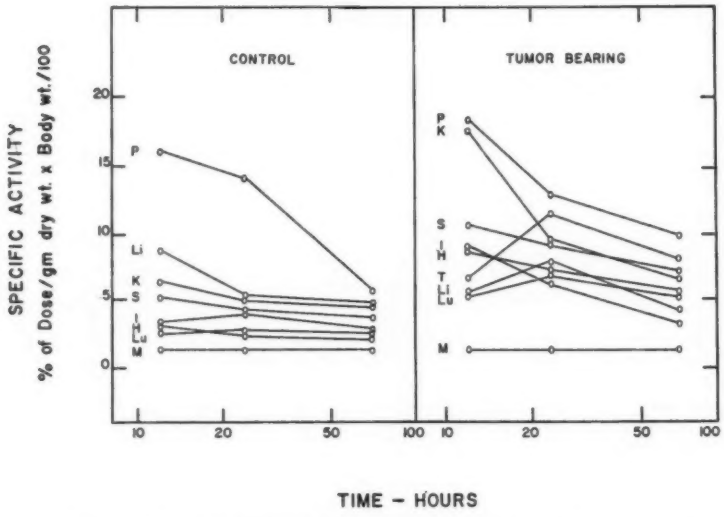
and the lowering of the maximum value obtained. In table 1 is tabulated cumulative excretion of C^{14} and of carbon dioxide. The magnitude of the excretion in the normal rats is similar to that observed by Borsook *et al.* (*8*) and by Soucy and Bouthillier (*9*). It is seen that although carbon dioxide production tends to be lower in the tumor-bearing animals the reduction in isotope clearance cannot be understood in terms of this factor alone. It may be mentioned that the specific activity of urinary urea obtained from these animals was also lower in those bearing tumors than the normals (*1*). This is to be expected since the average activity of the urea follows that of carbon dioxide (*10*).

Tissue distribution.—In text-figure 2 the isotope concentration in the tissues of major interest of both groups of rats is plotted against the logarithm of time. Although the data on which the graphs rest are not

TABLE 1.—*Excretion of radioactivity and carbon dioxide*

	Total C^{14} (% of dose)			Total CO_2 (millimoles)		
	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.
Normals:						
Rat A	20. 5	—	—	176		
Rat B	20. 4	21. 8	—	193	276	
Rat C	25. 0	—	35. 0	169	293	913
Tumor-bearing:						
Rat A	6. 30	—	—	151		
Rat B	6. 08	7. 13	—	164	296	
Rat C	5. 16	6. 27	7. 81	141	276	695

sufficiently extensive to provide a complete delineation of the time curves, they are sufficiently detailed to demonstrate that there are major abnormalities in host metabolism. It is seen that there is a general stimulation of isotope uptake in the tissues of the tumor-bearing host. The stimulation is greatest in the more active tissues, while in the case of muscle, which acquires only a comparatively low concentration, the effect is of doubtful significance. The differentials are most marked at 12 hours, when the concentration ratios between corresponding pairs of tissues are 2- to 3-fold. Later on the ratios drop to a range of 1.5 to 2.5.



TEXT-FIGURE 2.—Time relationships of tissue activity. P, plasma; Li, liver; K, kidney; S, spleen; I, intestine; H, heart; Lu, lung; M, muscle; T, tumor.

The rate of decrease of isotope concentration is distinctly greater in the host tissues than in those of the controls. Again, muscle does not show the effect. The levels attained by the control tissues at 12 hours hold the same relative rank throughout the experimental period with the exception of the crossover between lungs and heart, while in the tumor-bearing group there are numerous crossovers. Moreover, there is little resemblance between the rank taken up by the host and control tissues. There is a difference in the shape of the plasma curves for the 2 groups of rats, which suggests that in the tumor-bearing group the activity of the plasma reaches a peak earlier and falls more abruptly than it does in the controls. On the other hand, the tumor reaches peak activity much later than most of the other tissues represented. Finally, there is a noteworthy distortion of the curve for the liver in the host group. This tissue is unique in possessing a specific activity at 12 hours lower than its normal counterpart. It does not reach its maximum until more than 12 hours after administration of histidine, but attains a value higher than that of normal liver at this time.

By 72 hours the specific activity has fallen to a value below the normal. The slower rise of the host liver reminds one of the displacement of the carbon dioxide peak seen in text-figure 1, although the position of the maxima is not the same.

Table 2 shows the gross partition of isotope between the tissues and the various excreta. The data suggest that a substantial part of the extra isotope originally taken up by the tissues in the host animals is eliminated via the feces.

TABLE 2.—*Gross partition of isotope expressed as percentage of dose*

	Normal			Tumor-bearing		
	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.
Respiratory excretion	20. 5	21. 8	35. 0	6. 3	7. 1	7. 8
Urinary excretion	5. 5	9. 5	6. 8	2. 1	3. 1	7. 2
Fecal excretion	9. 3	8. 4	3. 5	8. 8	16. 2	11. 1
Total tissue incorporation	64. 7*	60. 3*	54. 7*	83. 5†	82. 0†	58. 9†

*By difference.

†A complete distribution study was done on the tumor-bearing group but data for tissues other than those in text-figure 2 have been omitted since they showed little of interest.

Amino acids.—A trace of isotope occurs in many of the amino acids but this could be the result of isotope leakage via reversible reactions. Since we are not interested in this process the glutamic and aspartic acid levels have been chosen as those below which we do not consider the data to be of interest. The dicarboxylic amino acids are chosen as the base line because it is known that they fix carbon dioxide by equilibration with intermediates of the Krebs cycle and the concentrations occurring in them may therefore be taken as a rough measure of the maximum values to be expected from carbon dioxide fixation alone. By this criterion the labeled carbon atom of histidine contributes significantly to the synthesis of methionine, serine, and cystine, and not to any of the others. The significant data are given in table 3.

Each of the significantly active amino acids requires formate for its biosynthesis and, since histidine is a formate precursor (*9,11–13*), we interpret the observed interrelationships in terms of formate metabolism. The methionine activity presumably arises from methyl-group formation. The operation of this process in the tumor appears to be essentially normal.

The serine activity, which has been observed before (*9,12*), arises by condensation of formate with glycine (*14*). That essentially all of the isotope is in the hydroxymethyl group as required by this mechanism was confirmed by periodate oxidation of samples obtained from each of the 3 types of tissue. Examination of the serine values in table 3 reveals a major biochemical abnormality of the DAB hepatoma. In this tumor the transport of isotope along this pathway is reduced to 5 to 10 percent of its magnitude in normal liver. A comparison with the values in host liver is of interest but unfortunately we experienced great difficulty in the chromatography of serine and also cystine on Dowex-50 and some

TABLE 3.—*Specific activity* of the amino acids from hepatic protein*

	Normal liver			Host liver			Tumor		
	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.
Aspartic acid	—	1. 7	1. 1	0. 9	1. 1	1. 7	—	0. 4	0. 6
Glutamic acid	3. 1	4. 6	2. 1	. 9	1. 9	. 6	4. 4	2. 7	0
Methionine	9. 17	13. 8	6. 44	18. 8	6. 83	9. 45	9. 70	21. 1	14. 4
Serine	64. 1	—	43. 1	42. 8	90. 4	—	4. 3	4. 9	5. 8
1/2 Cystine	23. 5	—	7. 99	18. 8	27. 0	—	5. 6	12. 5	8. 9
Histidine	1130	792	—	1460	1880	1060	1280	—	1660

*Expressed as dpm per $\mu M \times \frac{\text{body weight (gm.)}}{100} \times \frac{1}{\text{dose } (\mu C)}$.

samples did not survive the repeated runs necessary to effect purification. The available data indicate that the specific activity of serine in the host liver is markedly increased.

We reported earlier (*1*) that tumor choline takes up isotope in the methyl groups only, unlike choline isolated from normal and host liver where about one third of the total isotope is in the ethanolamine moiety. We interpreted this to mean that there is a block either in the condensation of formate with glycine or in the decarboxylation of serine. The serine data given here show that it is the former process which is involved.

The cystine activity presumably arises by reaction between serine and homocysteine (*15*), but it is not certain that this is the complete explanation. It is seen that in the normal and host livers the serine acquires a higher isotope concentration than does the cystine as is required if a precursor relationship is to be satisfied, and in the tumor the cystine level is reduced, as it should be since the serine level is reduced. However, in the tumor the cystine acquires a higher specific activity than does the serine. This suggests that at least some of the isotope enters the cystine molecule by a pathway which does not involve serine. Unfortunately, insufficiency of material precluded degradation studies.

Histidine is, of course, the most highly active of the amino acids, exceeding serine, which is next, by about 20-fold in normal liver. The histidine has undergone over-all biological dilution of about 1,000-fold. The time course of histidine activity follows that observed in text-figure 2 for gross tissue activity as would be expected. At 12 hours in both tumor and host liver the specific activity of histidine is the same as or a little higher than that in normal liver, but the gross isotope concentration is lower. This indicates that at this time a smaller proportion of the total radioactive material in normal liver is protein-bound. A similar observation has been reported for glycine (*16*) in rats bearing hepatoma induced by *N,N*-dimethyl-*p*-(*m*-tolylazo)-aniline.

Nucleotides.—The specific activities of the nucleotides are given in table 4. The isotope in the purine nucleotides is all present in the nitrogenous bases. This fact was established by degradation of various samples with hot hydrochloric acid and isolation of the ribose moiety as the 2,4-dinitrophenylhydrazone of furfural. The derivatives were devoid of radioactivity. Thus under our conditions we fail to observe the incorporation of formate into ribose reported by Bernstein (*17*). It is assumed that the same is true for the pyrimidine nucleotides.

There is some incorporation of isotope into the pyrimidines, but the major part is in the purines, in accord with the formate mechanism. We experienced some difficulty in obtaining pure cytidylic acid specimens by chromatography on Dowex-1 because of the persistence of an impurity of high activity. Rechromatography with a 0.01 *M* ammonium formate buffer at *p*H 4.0 was the best procedure we were able to devise, but in 2 cases pure specimens were not obtained and the values for cytidylic acid A at 12 hours and cytidylic acid B at 72 hours in normal liver may be somewhat high. The uridylic acids chromatographed well as a mixed

TABLE 4.—*Specific activity* of nucleotides and purines*

	Normal liver			Host liver			Hepatoma		
	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.	12 hr.	24 hr.	72 hr.
Adenylic acid A	60. 5	121	149	68. 8	408	353	25. 5	54. 4	—
Adenylic acid B	60. 8	104	158	65. 0	420	353	16. 0	45. 9	24. 3
Guanylic acid A	56. 0	25. 7	136	90. 0	576	408	17. 3	71. 2	15. 9
Guanylic acid B	58. 3	26. 1	132	97. 5	485	394	17. 3	51. 5	24. 6
Cytidylic acid A	5. 2	5. 6	3. 9	0	—	0	0	3. 7	—
Cytidylic acid B	1. 6	6. 3	7. 1	0	—	. 9	0	5. 5	—
Uridylic acids	5. 4	9. 7	2. 0	1. 9	8. 9	5. 7	5. 2	10. 3	5. 5
Adenosine-5-phosphate	320	337	—	204	906	—	420	—	161
DNA adenine	3. 4	5. 8	33. 0	6. 5	30. 8	68. 9	2. 1	—	—
DNA guanine	2. 4	24. 8	20. 6	21. 0	31. 8	130	7. 8	1. 2	—

* Units as in table 3.

peak of both isomers. The pyrimidine values show no significant differences between the 3 types of tissue.

The ribonucleic acid purines of normal liver exhibit a steady rise in isotope concentration up to 72 hours with equal concentrations in the A and B isomers and a similar magnitude in adenine and guanine as has been observed by others with various precursors. The 24-hour points for the guanylic acids appear to be out of line, but careful rechecking of the samples failed to produce a change in the values. In the host liver, guanine incorporation is somewhat favored relative to adenine, and there is a large increase in isotope concentration in both purines which in the case of adenine amounts to 3.7-fold at 24 hours. Attainment of peak activity occurs at some time between 12 and 72 hours, much earlier than in control liver.

In the tumor PNA there is a similar acceleration in the attainment of peak isotope concentration. On the other hand, the isotope concentration is drastically reduced and at no time does it approach the level of either normal or host liver. At 24 hours the tumor level is roughly 50 percent of that of normal liver and 10 percent of host liver. At 72 hours the factors are 15 percent and 5 percent, respectively.

The DNA purines of normal liver incorporate less isotope than those of PNA, in accord with general experience. The DNA, like PNA, increases its isotope concentration steadily up to 72 hours. In the host liver a stimulation of incorporation is seen again but the attainment of peak activity is slower than for PNA, being still incomplete at 72 hours. The same blockage of incorporation that occurs in PNA is seen in the tumor. We can say nothing about the time relationships in the tumor because the recovery from 3 of the tumors was too low to permit counting of the purine specimens.

We find that a small amount of adenosine-5-phosphate present in the tissues is not removed by cold trichloroacetic acid. This fraction was identified by its position on the chromatogram between the cytidylic acid peak and adenylic acid A and by demonstrating the presence of organic phosphate, ribose, and correct spectral properties. The quantity obtained is roughly 5 percent of the amount of either adenylic acid A or B. From 3 tissues, the amount of material obtained was insufficient for radioactivity assay. This is unfortunate because it destroys the picture of the time relationships. The data are sufficient to establish, however, that the isotope level in this nucleotide is much higher than in the adenine of PNA in all tissues. The same relationship exists when glycine is the labeled precursor (*18*). There appears to be a stimulation in the host liver, but it is difficult to judge what the situation is in the tumor. At 12 hours the level is higher in the tumor than in either normal or host liver. It would appear that the blockage of incorporation observed in PNA does not operate or is at least not so severe in adenosine-5-phosphate.

Discussion

Although no systematic tracer study of amino acid uptake in intact rats bearing DAB hepatoma has been reported, Griffin *et al.* (*16*) have investi-

gated the uptake of glycine-2-C^{14} in Sprague-Dawley rats bearing hepatoma induced by feeding the related carcinogen *N,N*-dimethyl-*p*-(*m*-tolylazo)-aniline (3′-methyl-4-dimethylaminoazobenzene, MDAB). Their data do not show a delay in the attainment of peak isotope concentration in the proteins of host liver, although there is a delay in the tumor of the same magnitude as we observe. They find that the isotope concentration in tumor protein is not greatly different from that in host liver, as do we.

In vitro, the protein isotope concentration was found by Zamecnik *et al.* (*19*) to rise steadily from normal liver to host liver to hepatoma upon incubation of glycine 1-C^{14} and DL-alanine-1-C^{14} with tissue slices from Harvard colony rats fed DAB. The increment in the hepatoma was about eightfold. Rutman *et al.* (*20*) found the same progression with DL-alanine-1-C^{14} in fortified mitochondria from rats bearing MDAB hepatoma although the tumor increment was only about twofold. Our data show the same progression if the comparison is made at 24 hours when the tumor and host liver are at their peak, although the differential is much less than that observed in the DAB studies *in vitro*. At 12 hours, however, there is essentially no difference and, since the liver proteins ordinarily reach peak activity within 2 or 3 hours after administration of a labeled amino acid, there is no doubt that at this time the progression is inverted. Thus one sees that comparisons of this kind are meaningless unless made with reference to the time relationships.

A curious feature of the experiments *in vitro* is that this inversion does not occur; tumor and host-liver increments are apparent from the start and are progressively magnified up to the end of the comparatively short periods that can be studied. These facts indicate that the mechanism of incorporation into the tumor proteins *in vitro* is somewhat different from that *in vivo*. In particular, the delayed peak *in vivo* suggests that plasma protein plays a commanding part in the transport of histidine to the tumor, since it is difficult to discern any other source which at 12 hours contains isotope at a specific activity sufficiently high that donation to the tumor will produce a still rising level. This idea is supported by the data of Griffin *et al.* (*16*) for glycine in the MDAB hepatoma where at 2 hours 70 percent of the radioactivity in the tumor is in the proteins, while only 13 percent of the liver activity is protein-bound. We have already noted in a qualitative way that a similar situation obtains in the DAB animals. Busch and Greene (*21*) have recently made a similar suggestion after finding that tumor protein becomes labeled more strongly than protein of other tissues when labeled plasma protein is administered but takes up much less isotope (at 6 hours) when a free amino acid is given. They used the Walker and Flexner-Jobling tumors.

Shifts in the protein-uptake curve of host liver and other viscera similar to those we observe have been reported by Norberg and Greenberg (*22*), who studied the metabolism of glycine-1-C^{14} in mice bearing the transplanted Gardner lymphosarcoma. The peak isotope levels attained by plasma, liver, and spleen of the host were above normal. In addition, their data show a distinct delay in the liver peak although it is not so

pronounced as the histidine delay in DAB liver. Wase *et al.* (*23*) found a liver enhancement after administration of labeled DL-methionine to rats fed *N*-2-fluorenylacetamide (2-acetylaminofluorene). On the other hand, LePage and Heidelberger (*24*) studying glycine uptake by rats bearing multiple Flexner-Jobling implants found no effect on protein incorporation in the host.

The change in the time relationships governing incorporation into PNA purines of hepatoma and host liver appears to involve factors different from those concerned in the disturbance of protein metabolism, since these tissues show an acceleration instead of a retardation in the attainment of peak activity. The enhancement of peak activity exhibited in host liver is presumably to be expected under conditions of accelerated incorporation but the profound reduction in the tumor level indicates an additional effect. Since the total PNA concentration in the hepatoma is not greatly different from that in normal liver (*25*), it does not appear likely that the low isotope concentration of the PNA purines indicates a reduced synthesis of these materials. Rather it seems that formate incorporation is blocked, and a larger proportion of the tumor's requirement is met in a different way, presumably with preformed purines obtained from the diet or the host. Support for this idea is found in the lack of a depression of the isotope concentration in the nucleic acids of MDAB hepatoma after administration of labeled phosphate (*26*) or adenine (*27*).

Blockage of formate incorporation might occur in two ways:

1) There might be an impairment of formate production. Indeed a low histidase content has been reported in DAB hepatoma by Masayama *et al.* (*28*) and by Viollier (*29*). However, they also found a lowered activity in host liver, but incorporation is stimulated in this tissue. It is apparent that the measurements of the histidase activity *in vitro* do not give a quantitative measure of its activity *in vivo*. Such a situation is not uncommon. Moreover, the isotope level in tumor methionine is not reduced and that in the methyl groups of tumor choline, while exhibiting a lag, rises at 72 hours to a value as high as that attained in normal liver (*1*). Simple reduction of the formate supply should presumably depress to a similar extent both methyl-group formation and formate-condensation reactions.

2) There might be a blockage of formate condensation *per se*. It is this interpretation which we favor, although some degree of impairment of the formate supply is perhaps not excluded. Presumably it is some part of the folic acid system which is involved, and it would appear that a defect in this mechanism causes a shift of the favored pathway for PNA synthesis in the tumor away from formate and toward other precursors. The possibility of such a shift is of obvious interest in connection with the phenomenon of differing degrees of resistance or susceptibility exhibited by various types of tumors toward chemotherapeutic agents designed to interfere with purine and nucleic acid metabolism.

Little can be said about the mechanism responsible for the disturbances

in the viscera but in this connection it is of interest to inquire whether there is a direct causal relationship between the reduced oxidation of histidine to carbon dioxide by the DAB rat and the increased tissue incorporation; that is, whether an impairment of the capacity for amino acid oxidation causes a piling up which leads to increased protein and nucleic acid synthesis or, alternatively, whether increased anabolic needs cause a diversion away from the oxidative pathway. There is at present no good basis for a choice between the alternatives. If the former is valid, however, it may be a clue to an understanding of some of the effects of a tumor on host metabolism.

Summary

Rats bearing hepatoma induced by *N,N*-dimethyl-*p*-phenylazoaniline (*p*-dimethylaminoazobenzene), when fed L-histidine-2-C^{14} convert less of the labeled carbon to carbon dioxide and incorporate more into visceral protein than do normal controls. The possibility of a causal relationship between the effects is suggested. The isotope concentration in nucleic acid purines of the tumor is below normal, indicating a possible impairment of the folic acid system. The level in tumor protein is above normal. Host liver exhibits an enhancement of the levels in both nucleic acid and protein. Time relationships indicate that the tumor utilizes histidine mainly from plasma protein rather than as the free amino acid. Conversion of labeled carbon to other amino acids is limited to those whose biosynthesis involves formate.

References

(*1*) Reid, J. C., Landefeld, M. O., and Simpson, J. L.: Metabolism of L-histidine-2-C^{14} in the normal and hepatoma-bearing rat. J. Nat. Cancer Inst. 12: 929–936, 1952.

(*2*) Daoust, R.: Quantitative histology of rat liver during carcinogenesis by *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 15: 1447–1449, 1955.

(*3*) Schmidt, G., and Thannhauser, S. J.: A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161: 83–89, 1945.

(*4*) Cohn, W. E.: The anion-exchange separation of ribonucleotides. J. Am. Chem. Soc. 72: 1471–1478, 1950.

(*5*) Schneider, W. C.: Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161: 293–303, 1945.

(*6*) Stein, W. H., and Moore, S.: Chromatographic determination of the amino acid composition of proteins. Cold Spring Harbor Symp. Quant. Biol. 14: 179–190, 1949.

(*7*) Moore, S., and Stein, W. H.: Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem. 192: 663–681, 1951.

(*8*) Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H.: Metabolism of C^{14}-labeled glycine, L-histidine, L-leucine, and L-lysine. J. Biol. Chem. 187: 839–848, 1950.

(*9*) Soucy, R., and Bouthillier, L. P.: The metabolic fate of atom 2 of the imidazole ring of histidine studied with the aid of radiocarbon. Rev. Canad. biol. 10: 290–291, 1951.

(*10*) MacKenzie, C. G., and du Vigneaud, V.: The source of urea carbon. J. Biol. Chem. 172: 353–354, 1948.

(*11*) Reid, J. C., and Landefeld, M. O.: Synthesis of purines and methyl groups from carbon atom 2 of histidine. Arch. Biochem. 34: 219–221, 1951.

(*12*) Sprinson, D. B., and Rittenberg, D.: The metabolic reactions of carbon atom 2 of L-histidine. J. Biol. Chem. 198: 655–661, 1952.

(*13*) Toporek, M., Miller, L. L., and Bale, W. F.: Carbon atom 2 of L-histidine-2-C^{14} as a source of the carbon of labile methyl groups in the liver. J. Biol. Chem. 198: 839–851, 1952.

(*14*) Sakami, W.: The conversion of formate and glycine to serine and glycogen in the intact rat. J. Biol. Chem. 176: 995–996, 1948.

(*15*) Binkley, F., and du Vigneaud, V.: The formation of cysteine from homocysteine and serine by liver tissue of rats. J. Biol. Chem. 144: 507–511, 1942.

(*16*) Griffin, A. C., Bloom, S., Cunningham, L., Teresi, J. D., and Luck, M.: The uptake of labeled glycine by normal and cancerous tissues in the rat. Cancer 3: 316–320, 1950.

(*17*) Bernstein, I. A.: Synthesis of ribose by the chick. J. Biol. Chem. 205: 317–329, 1953.

(*18*) Edmonds, M. P., and LePage, G. A.: The incorporation of glycine-2-C^{14} into acid soluble nucleotide purines. Cancer Res. 15: 93–99, 1955.

(*19*) Zamecnik, P. C., Frantz, I. D., Jr., Loftfield, R. B., and Stephenson, M. L.: Incorporation in vitro of radioactive carbon from carboxyl-labeled DL-alanine and glycine into proteins of normal and malignant rat livers. J. Biol. Chem. 175: 299–314, 1948.

(*20*) Rutman, R. J., Cantarow, A., and Paschkis, K. E.: Studies in 2-acetylaminofluorene carcinogenesis. II. The *in vitro* uptake of alanine-1-C^{14} by preneoplastic liver and hepatoma mitochondrial protein. Cancer Res. 14: 115–118, 1954.

(*21*) Busch, H., and Greene, H. S. N.: Studies on the metabolism of plasma proteins in tumor-bearing rats. Yale J. Biol. & Med. 27: 339–349, 1955.

(*22*) Norberg, E., and Greenberg, D. M.: Incorporation of labeled glycine in the proteins of tissues of normal and tumor-bearing mice. Cancer 4: 383–386, 1951.

(*23*) Wase, A. W., Allison, J. B., and Migliarese, J. F.: The effect of 2-aminofluorene on the utilization of S^{35}-DL-methionine. Science 116: 536–537, 1952.

(*24*) LePage, G. A., and Heidelberger, C.: Incorporation of glycine-2-C^{14} into proteins and nucleic acids of the rat. J. Biol. Chem. 188: 593–602, 1951.

(*25*) Price, J. M., Miller, J. A., Miller, E. C., and Weber, G. M.: Studies on the intracellular composition of liver and liver tumor from rats fed 4-dimethylaminoazobenzene. Cancer Res. 9: 96–102, 1949.

(*26*) Griffin, A. C., Cunningham, L., Brandt, E. L., and Kupke, D. W.: Effect of a carcinogenic azo dye on radiophosphorus turnover in rat-liver nuclei and cytoplasm. Cancer 4: 410–415, 1951.

(*27*) Griffin, A. C., Davis, W. E., Jr., and Tifft, M. O.: The liver nucleic acid incorporation of adenine-8-C^{14} during azo dye carcinogenesis. Cancer Res. 12: 707–712, 1952.

(*28*) Masayama, T., Iki, H., Yokoyama, T., and Hasimoto, M.: Biochemical study during the course of liver cancer production by dimethylaminoazobenzene feeding. Gann 32: 303–305, 1938.

(*29*) Viollier, G.: On the enzyme content of benign and malignant liver tumors. I. Arginase and histidase. Helv. physiol. et pharmacol. acta 8: C34–C36, 1950.

Modification of Treatment Schedules in the Management of Advanced Mouse Leukemia with Amethopterin [1]

ABRAHAM GOLDIN, JOHN M. VENDITTI, STEWART R. HUMPHREYS, *and* NATHAN MANTEL, *Laboratory of Chemical Pharmacology and Biometry and Epidemiology Branch, National Cancer Institute,*[2] *Bethesda, Maryland*

Previous studies (*1–4*) have demonstrated that periodic and continued treatment with the folic acid antagonists aminopterin and amethopterin provides a certain amount of success in the management of the transplanted mouse leukemia L1210. Though only a limited proportion of complete tumor regressions was achieved, the survival times of the leukemic mice were greatly enhanced, and there was some indication that so long as treatment was continued the mice could be kept alive and free of tumor. With continued treatment, of course, there remained a continuing risk that the mice would succumb to the cumulative toxic effects of therapy.

A limitation of this work was that it applied to the case where therapy was instituted relatively early in the course of the disease, generally within 4 days after tumor implantation. While continuing treatment with amethopterin every 4 days appeared to be optimal when therapy was instituted early, there was no implication that any success could be achieved in treating an advanced leukemia. In fact, earlier work (*1*) had shown that single treatment with aminopterin was virtually ineffective when applied 9 days following implantation. Since in any program of therapy one cannot be selective about the stage of the disease one encounters, it becomes important to develop therapies for both advanced and early stages of the disease.

The antileukemic effectiveness of the antifolics may be altered by factors pertaining to both the tumor and the host (*1,2,4–8*). Drug effectiveness was influenced by factors such as: *a*) time at which treatment was initiated; *b*) spacing of the treatments; *c*) number of treatments; *d*) administration of a metabolite; *e*) concentration of leukemic inoculum; *f*) age and weight of the mice. The current experiments were conducted to determine whether the optimal schedule of treatment with a drug is dependent upon the stage of the disease. Also, it was considered desirable to determine the feasibility of treating advanced leukemia in mice.

---

[1] Received for publication April 6, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Methods

The 2 experiments reported here were conducted in 9- to 12-week-old CDBA[3] hybrid male mice inoculated in the right thigh with a uniform suspension of leukemic cells (L1210). Stock tumor was taken from DBA/2J mice. A measured concentration of leukemic cells was employed in all experimental and appropriate control groups. In addition, in each experiment, the leukemic mash was serially diluted and inoculated into CDBA mice in order to test the potency of the inoculum. The mice were completely randomized in the prescribed injection groups. Amethopterin[4] was dissolved in 2 percent sodium bicarbonate and administered intraperitoneally to the mice in the constant volume of 0.01 ml. per gm. The dosage schedules are indicated with the individual experiments.

In earlier studies (*1,5,6*) involving treatment of limited duration, advantage was taken of a temporal separation of toxic and tumor deaths in comparing alternative therapies. The better therapy was the one yielding the higher survival time for a specified percentage of toxic mortality. In studies such as the present one, where extensive treatment schedules are employed, the separation of toxic and tumor deaths becomes difficult, especially since animals may succumb to a combination of drug toxicity and tumor growth.

For purposes of evaluating a particular therapeutic schedule, it is necessary (if no attempt is made to separate toxic deaths from tumor deaths) to determine the optimal dose for that schedule. If the dose is too low, animals will die early because of growth of the tumor; if it is too high, animals will succumb to the toxicity of treatment. An optimal dose for a schedule can be considered to be that dose at which death of the animals is retarded longest, no matter what the cause of death.[5] The best of alternative schedules is considered to be that one in which the optimal dose elicited the longest survival time, for deaths from any cause. The current experiments were designed to permit this type of comparison.

Results

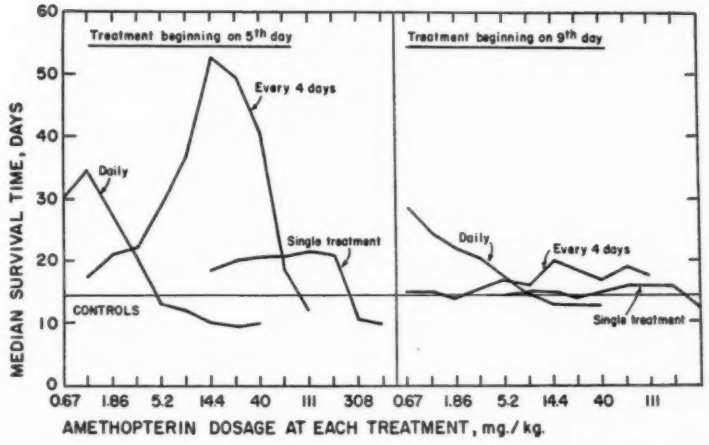
Text-figure 1 summarizes the results of the first experiment. Three different schedules of treatment with amethopterin were employed, starting at 5 and 9 days following inoculation of the leukemia. The 3 schedules were: single treatment, daily treatment, and treatment every 4 days. Treatment was continued until 103 days after tumor inoculation, at which time 4 mice were still alive.

For each schedule of treatment a series of dose levels of amethopterin was administered. Text-figure 1 shows the median survival time of the leukemic mice for each schedule and dose level. Typically, for each

[3] (BALB/cAn × DBA/2J)F_1.

[4] Amethopterin was kindly provided by Dr. James M. Smith, of the Calco Chemical Division of the American Cyanamid Company, Bound Brook, N. J.

[5] There can be alternative definitions of optimal doses. If complete regression of the tumor could be accomplished with reasonable frequency, an optimal dose could be one yielding the largest proportion of animals alive and free of tumor—deaths from any cause, or at any time, being considered therapeutic failures.



TEXT-FIGURE 1.—Effect of schedule of treatment with amethopterin on the median survival time of mice with leukemia L1210. When treatment was initiated on the 5th day following tumor implant, the every-4-day schedule of treatment was most effective. When treatment was delayed until the 9th day, daily treatment became relatively more effective. The tumor inoculum was 1.13×10^5 cells per mouse, or 10 times that yielding 100 percent takes in the tumor titration.

schedule, the median survival time was low at the lower doses, increased with dose to a peak, and then decreased again. The sole exception in the data here is the daily treatment schedule beginning on day 9, and this resulted from the limited range of doses used. At the lowest dose of amethopterin used (0.67 mg./kg. daily) for this schedule, most of the mice died with substantial tumors. This indicates that further reduction of the dose level would have resulted in less extensive inhibition of tumor growth and diminished median survival time.

The controls in this experiment died with a median survival time of 14.5 days (table 1, cols. 3 to 6). The greatest extensions in survival time, for each schedule of treatment, were achieved by initiating treatment on day 5 (text-fig. 1, table 1). For this day of initial treatment, the every-4-day schedule of treatment yielded the optimal dose with the highest median survival time. The optimal dose here was 13 times the optimal dose for daily treatment. Expressed in terms of equivalent daily dose, it was more than 3 times as great as the optimal dose for daily treatment (table 1, cols. 1 and 2). The median survival time was raised to 52.5 days, representing a 262 percent increase.

For initial treatment on day 9, the optimal dose for daily treatment yielded a higher median survival time than did the optimal dose for treatment every 4 days. In this case, the optimal dose for daily treatment was less than one twentieth, and the equivalent daily dose less than one fifth, of the optimal dose and equivalent daily dose, respectively, for treatment every 4 days. Starting treatment on day 9, then, in contrast to day 5, the more effective therapy was accomplished with a reduced

TABLE 1.—*Influence of schedule of treatment on median survival time of mice with leukemia L1210. Characteristics of dosage yielding the greatest median survival time for each schedule of treatment*

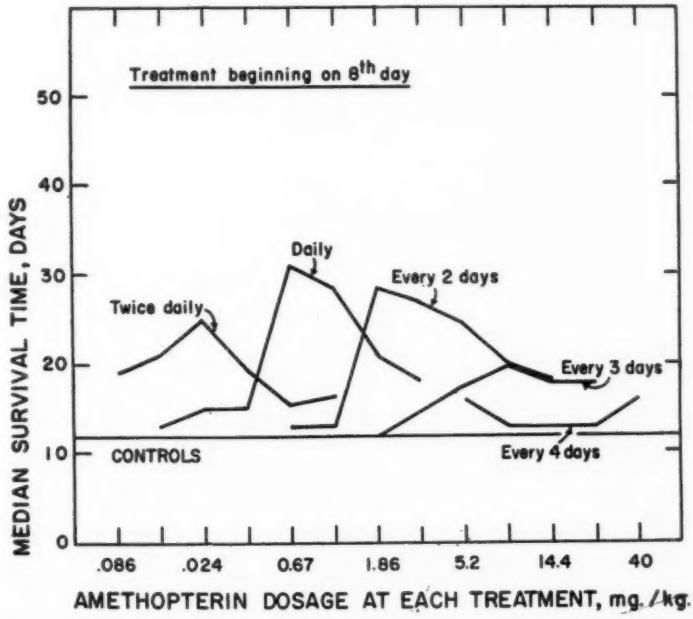
	Optimal dosage level (*mg./kg.*)	Equivalent daily dose (*mg./kg./day*)	Median survival time (*days*)	Relative survival time; untreated controls equal 100%	Median remaining lifetime; day of treatment equals 0	Relative remaining lifetime; untreated controls equal 100%
	(1)	(2)	(3)	(4)	(5)	(6)
First experiment						
Treatment beginning on day 5						
Single treatment	111	—	21. 5	148	16. 5	174
Daily treatment	1. 11	1. 11	34. 5	238	29. 5	311
Treatment every 4 days	14. 4	3. 6	52. 5	362	47. 5	500
Controls	—	—	14. 5	100	9. 5	100
Treatment beginning on day 9						
Single treatment	67–185	—	16	110	7	127
Daily treatment	0. 67	0. 67	28. 5	197	19. 5	355
Treatment every 4 days	14. 4	3. 6	20	138	11	200
Controls (same controls as above)	—	—	14. 5	100	5. 5	100
Second experiment						
Treatment beginning on day 8						
Treatment twice daily	0. 24	0. 48	25	208	17	425
Daily treatment	0. 67	0. 67	31	258	23	575
Treatment every 2 days	1. 86	0. 93	28. 5	238	20. 5	512
Treatment every 3 days	8. 6	2. 9	20	167	12	300
Treatment every 4 days	5.2 & 40	1.3 & 10	16	133	8	200
Controls	—	—	12	100	4	100

dose. The median survival time for mice receiving the optimal daily treatment was raised to 28.5 days, representing a 97 percent increase over untreated controls.

Single treatment with amethopterin on day 5 was less effective than the multiple treatments started at that time. Single treatment on day 9 was the least effective of all the treatments employed, giving only a 10 percent increase in survival time.

Thus, daily treatment was superior to treatment every 4 days, when initial treatment was delayed to the 9th day. Could some other interval between treatments be superior even to daily treatment for a late tumor? Text-figure 2 summarizes the results of an experiment on this point. Five different treatment intervals were tested against an 8-day-old tumor, the median survival time for untreated controls being 12 days. The treatment intervals were: twice daily, daily, every 2 days, every 3 days, and every 4 days. For each treatment interval, a series of dose levels was applied. Treatment was continued until 39 days after tumor inoculation, at which time 3 mice were still alive.

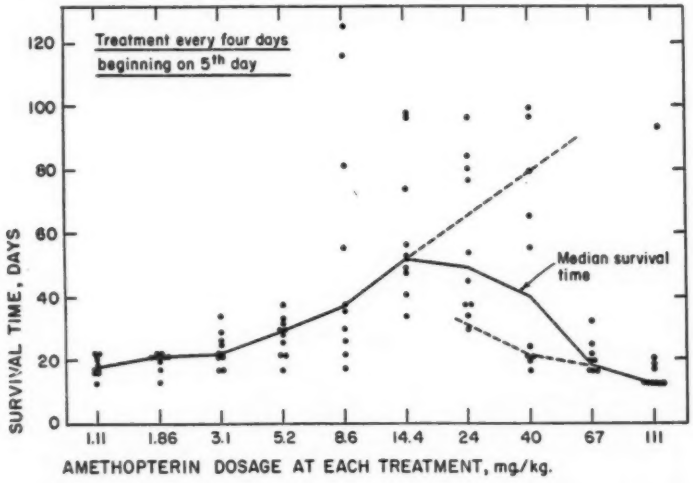
As can be seen from text-figure 2, daily treatment yielded the optimal dose with the highest median survival time. Apparently, then, for late nitiation of treatment, the daily schedule still seems to be optimal. The



TEXT-FIGURE 2.—Effect of interval between treatments with amethopterin on the median survival time of mice with leukemia L1210. With initiation of treatment delayed until the 8th day following tumor implant, a 1-day interval between treatments was most effective. The tumor inoculum was 1.6×10^6 cells per mouse, or 100 times that yielding 100 percent takes in the tumor titration.

optimal daily dose was the same as in the previous experiment (0.67 mg./kg.). This dose increased the median lifetime of the mice from 12 days, for untreated controls, to 31 days (table 1)—an increase of 158 percent. As can be seen from table 1, columns 1 and 2, ordinarily the more frequent the treatment, the lower the optimal dose level and average daily dose level. That "overtreatment" may occur is indicated by the results for the "twice-daily" schedule. The continued reduction in average daily dose was accompanied by a decrease, rather than a further increase, in survival time.

In the 2 experiments described here it was possible to test only a limited number of dose levels. There was no assurance that the optimal dose level found would be near and give results comparable to the very best dose level that could have been used. Relative to this point it is instructive to examine text-figure 3. Text-figure 3 presents the individually detailed days of death and median survival times at each level of treatment for the groups receiving treatment every 4 days starting on day 5 in the first experiment reported here.



TEXT-FIGURE 3.—(*cf.* text-fig. 1, first panel, treatment every 4 days)—Individual survival times and median survival time for mice with leukemia L1210 treated every 4 days with amethopterin. Treatment was initiated 5 days following tumor implantation.

The median survival times (text-fig. 3) first rise and then fall with dose. At the lower dose levels it would seem that there were few, if any, toxic deaths. This follows from the early occurrence of death at these low dose levels, most of the mice having large palpable tumors at the site of leukemic inoculation. The mice, in general, gained weight or failed to lose weight. Had there been toxic deaths at these low dose levels they should have occurred late, certainly later, say, than the earliest deaths occurring at the dose level of 14.4 mg. per kg. Similarly, the early deaths

at the high dose levels must be ascribed to toxicity. The mice lost weight and showed little or no evidence of palpable tumor at the site of inoculation. At these high dose levels any tumor deaths which occurred should have been at least as late as the tumor deaths at lower dose levels. At the intermediate dose levels, 24 and 40 mg. per kg., there appears to be a dichotomy in the times of death—there are early deaths consistent with the time at which toxic deaths would occur and late deaths consistent with the time at which tumor deaths would occur.[6] The time of tumor death could continue to increase indefinitely with dose at dose levels beyond the optimal dose found—however, actually only a limited increase in survival time from all causes could be obtained, due to the sharp rise in toxicity. If the rate of increase of survival time with dose is extrapolated beyond the optimal dose of 14.4 mg. per kg., as indicated in text-figure 3, it is seen that only a minor extension could result for doses intermediate between this and the next higher dose level. The survival times for the optimal dose levels found in these experiments can then safely be considered to be close approximations to the survival times which the very best dose level would have yielded, if administered.

In these experiments a total of 10 mice survived beyond the 90th day, but only a single live cure was achieved. This failure to achieve cures is felt to be due principally to the prolonged continuation of treatment. The levels of treatment which could have been successful in achieving cures were too high for the mice to endure indefinitely, and eventually they succumbed to the chronic toxicity of treatment. Many of these animals dying late showed no evidence of tumor and might well have survived had treatment been discontinued sooner.

A check on the stage of tumor infiltration was provided by implanting the blood and spleen of mice with 5- and 9-day-old tumors into normal mice of the same strain and sex. The mice with 5-day-old tumors showed no palpable tumors at the site of leukemic inoculation. For these mice, none of 10 blood implants and only 1 of 10 spleen implants resulted in subsequent tumor growth. Thus, a systemic leukemia capable of being retransplanted was substantially absent 5 days following tumor inoculation. The mice with 9-day-old tumors generally exhibited palpable tumors on the 9th day—for these mice, all 10 blood implants and all 10 spleen implants produced large local tumors which were lethal to their hosts. A similar check on the 8-day-old tumors of the 2nd experiment reported here showed that all 10 blood implants and all 10 spleen implants produced local tumors. At this later stage, there is present a systemic leukemia capable of being retransplanted.

Discussion

The current experiments illustrate the principle that, in the treatment of neoplasia, the optimal schedule of therapy and the optimal dose are not

[6] The actual cause for these late deaths is not clear. Many animals died late without tumor and with evidence of toxicity. For purposes of evaluating effectiveness of therapy they may, conservatively, be treated as tumor deaths, for they constitute evidence that treatment was effective in keeping the tumor in check for at least as long as the animal actually lived.

fixed, but may be influenced by the stage of development of the disease. With amethopterin, employing the transplanted leukemia L1210 in mice, in the early stages it was advantageous to use a long interval between treatments, the level of treatment being relatively high. When treatment was initiated after the leukemia had become frankly systemic, in this case only a few days prior to the death of untreated controls, daily low-level treatment became more effective than the high-level treatment every 4 days. Presumably, at other stages of the disease, other schedules and levels of treatment would have been optimal. These results emphasize the dual need in chemotherapy for recognition of the stage of disease and for the development of appropriate therapies for each stage.

A further result of these experiments was the demonstration that the advanced tumor is susceptible to therapy. In treating the early tumor, the effective therapy lies in applying large doses of drug, with a sufficiently long interval between doses to give the host a chance to recover. The comparatively successful therapy, in these studies, of the advanced tumor would seem to lie in the high frequency of treatment coupled with an extremely low dose level. The latter is so low as to be virtually non-damaging to the host, and the interval between treatments just long enough for the host to recover. The tumor itself may not recover from drug toxicity, but new growth of the tumor may more than compensate for damage inflicted by the drug and eventually the tumor grows large enough to cause the death of the host. Means for inflicting greater damage to the tumor than new growth can compensate have yet to be found.

It should be noted that in the current experiments the levels of therapy which were effective in treatment of advanced tumor were so low as to produce relatively little early weight loss or toxicity. In addition, in other experiments in which food-intake records were kept and in which similar low levels of drug were administered it was noted that the food intake, as well as the weight of mice, was maintained at high levels for a substantial period (*9*). Eventually, however, the chronic toxicity of treatment coupled with the continued growth of tumor resulted in serious reductions in food intake and animal weight. The food consumption of untreated mice dropped off early, but because of the rapid growth of tumor, total animal weight, including tumor, actually increased. These results clearly indicate that the effect of the drug against the tumor was not achieved indirectly through reduction of dietary intake caused by treatment.

Where the stage of the tumor is not known, the problem arises as to whether to treat it like an early or like an advanced tumor. Studies now are under way investigating the effectiveness of schedules which combine the infrequent high doses which are effective against early tumors with the frequent low doses which are effective against late tumors. Such a schedule, if found, may be near the optimal for both early and advanced tumors.

The effectiveness of therapy is ordinarily evaluated in terms of the survival time relative to that for untreated controls (table 1, col. 4). By this criterion late treatment is less effective than early treatment. An

alternative criterion, which corrects for differences in time of initiation of treatment, is the remaining lifetime, from the time of initiation of treatment, relative to that for untreated controls. For the current experiments this is shown in table 1 (cols. 5 and 6). In the first experiment reported here, untreated controls had a median survival time of 14.5 days or, for initial treatment on day 5, the remaining lifetime was 9.5 days. The optimal treatment every 4 days increased the median survival time to 52.5 days, or the median remaining lifetime to 47.5 days, representing a fivefold increase in the remaining lifetime. Comparable increases in the remaining lifetime for the optimal schedules were found for treatment beginning on day 9 in this experiment, and for treatment beginning on day 8 in the second experiment when the remaining lifetime was only 4 days. This concept for measuring effectiveness of therapy may be useful in studies involving several control groups each with varying survival time.

Summary

In previous studies, single treatment with antifolics, administered late in the course of the disease, was unsuccessful in prolonging the survival time of mice with the transplanted leukemia L1210. The present studies demonstrate that such advanced tumors are susceptible to therapy if the appropriate modified schedule of treatment with amethopterin is applied. In contrast to heavier and less frequent treatment with amethopterin when the tumor is in its earlier stages, the optimal schedule of treatment, when therapy is initiated late, is one in which very light doses of amethopterin are administered rather frequently. These results are discussed relative to the damage inflicted by the drug on the tumor and on the host.

References

(*1*) Goldin, A., Mantel, N., Greenhouse, S. W., Venditti, J. M., and Humphreys, S. R.: Factors influencing the specificity of action of an antileukemic agent (aminopterin). Time of treatment and dosage schedule. Cancer Res. 14: 311–314, 1954.

(*2*) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., Mantel, N., and Greenhouse, S. W.: Factors influencing the specificity of action of an antileukemic agent (aminopterin). Multiple treatment schedules plus delayed administration of citrovorum factor. Cancer Res. 15: 57–61, 1955.

(*3*) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., and Mantel, N.: Studies on the management of mouse leukemia (L1210) with antagonists of folic acid. Cancer Res. 15: 742–747, 1955.

(*4*) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., Mantel, N., and Greenhouse, S. W.: A quantitative comparison of the antileukemic effectiveness of two folic acid antagonists. J. Nat. Cancer Inst. 15: 1657–1664, 1955.

(*5*) Goldin, A., Mantel, N., Greenhouse, S. W., Venditti, J. M., and Humphreys, S. R.: Estimation of the antileukemic potency of the antimetabolite aminopterin, administered alone and in combination with citrovorum factor or folic acid. Cancer Res. 13: 843–850, 1953.

(*6*) ———: Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of aminopterin in mice. Cancer Res. 14: 43–48, 1954.

(7) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., DENNIS, D., and MANTEL, N.: Factors influencing the specificity of action of an antileukemic agent (aminopterin): Host age and weight. J. Nat. Cancer Inst. 16: 709–721, 1955.

(8) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., and MANTEL, N.: The influence of the concentration of leukemic inoculum on the effectiveness of treatment. Science, 1956. In press.

(9) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., and MANTEL, N.: Unpublished data.

Urinary Excretion of Corticosteroids in Guinea Pigs With malignant Neoplastic Diseases [1,2]

ELI M. NADEL *and* SHLOMO BURSTEIN,[3,4] *Laboratory of Pathology, National Cancer Institute,*[5] *Bethesda, Maryland, and The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts*

The nature of the relationship between the adrenal glands and neoplastic disease has been the subject of numerous investigations covering a wide range of approaches to the problem. *Clinically*, studies on urinary excretion have been held as a particularly hopeful approach (*1–5*). However, little conclusive information exists to date on the corticosteroid secretory response to neoplastic diseases originating outside of the pituitary or adrenal glands (*6*). This may be due partly to the extreme difficulty in establishing reliable control data in man. *Experimentally*, there are no reports on the urinary excretion of corticosteroids in animals with malignant neoplastic disease. Such studies are technically difficult in the mouse or the rat, mammals most frequently used in cancer studies, and little is known about the nature of their adrenocortical excretion. With the availability of 2 transplantable tumors (*7,8*) in inbred guinea pigs (*9*), the controlled study of the effect of 2 malignant neoplastic diseases (leukemia and liposarcoma) on the excretion of corticosteroids in this animal became feasible, since, as has been previously demonstrated, the guinea pig excretes in the urine large amounts of easily measurable corticosteroids (*10–17*). Several of these have been characterized by isolation and identification studies. Of these, the 3 major urinary unconjugated (free) reducing corticosteroids have been shown to be *cortisol* (*12*), *6β-hydroxycortisol* (*13,14*), and the recently identified *2α-hydroxycortisol* (*15*) [previously described as Steroid IIa (*13,14*)]. A method by which these 3 constituents could be separately determined by the use of chromatographic fractionation has been described (*14*).

It is the purpose of this study to report on the excretion of the free reducing steroids—*cortisol*, *2α-hydroxycortisol*, and *6β-hydroxycortisol*—in normal and in tumor-bearing strain 2 guinea pigs, before and after the administration of ACTH.

---

[1] Received for publication April 23, 1956.

[2] Supported in part by Research Grant No. NSF-G664, National Science Foundation.

[3] Visiting Scientist, National Cancer Institute.

[4] The authors wish to thank Arthur G. Hilgar, Bobby G. Young, and Miss Arline M. Tillotson for their technical assistance. The constant interest and encouragement of Dr. Harold L. Stewart and Dr. Ralph I. Dorfman are gratefully acknowledged.

[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Methods

I. Biological

A. *Animals.*—Strain 2 guinea pigs used throughout this experiment were supplied by the Animal Section, Laboratory Aids Branch, National Institutes of Health. Animals were of either sex, approximately 3 to 4 months old, and weighed between 350 and 450 gm. at the beginning of the experiment. Strain 2 guinea pigs have been inbred for over 70 generations of brother × sister matings (*9*).

B. *Diet.*—Purina rabbit chow and water were fed daily *ad libitum.* Cabbage was offered 6 times weekly and carrots once a week.

C. *Transplantable tumors.*—Leukemia L2B, an acute leukemia in its 22nd transplant generation, was provided by the late Dr. Egon Lorenz (*7*). Liposarcoma D4888, in its 107th transplant generation, was generously supplied by Dr. Walter Heston (*8*). Both tumors have been maintained in transplant for over 4 years in the Laboratory of Pathology, National Cancer Institute.

Leukemia L2B arose in a guinea pig that had been exposed to whole-body X irradiation. Liposarcoma D4888 arose in a guinea pig that had received an intramuscular injection of methylcholanthrene. These tumors have been successfully transplanted and have grown progressively in strain 2 guinea pigs. After transplantation of the leukemia, death occurs usually between the 14th and 20th days, and between the 28th and 39th days following transplantation of the liposarcoma.

D. *Transplantation.*—Leukemia L2B was transplanted subcutaneously into the right axilla by using a glass trocar and a small mass of tumor tissue or spleen, or by using a 1:1 brei of splenic and tumor tissue in Locke's solution in a syringe. Liposarcoma D4888 was transplanted subcutaneously to the right axilla by a trocar implant of tumor tissue.

E. *Pathologic study.*[6]—Complete autopsies, including the brain, were done on all guinea pigs. Tissues were fixed in 10 percent neutral formalin embedded in paraffin, and stained with hematoxylin and eosin. All animals receiving a transplant of leukemia died with leukemia confirmed by gross and histopathologic *post-mortem* examination. All animals receiving a transplant of liposarcoma died with liposarcoma confirmed by gross and histopathologic *post-mortem* examination.

F. *Urine collection.*—Seventeen-hour urinary samples were collected in special metabolism cages[7] which effectively separated urine from feces. Animals were caged individually and urines were collected individually, but, in order to have a sufficient quantity for isolation and quantitative analysis, it was necessary to pool urines from similarly treated animals as had been done in previous studies (*14*). The numbers of individual

---

[6] The results of the clinical and pathologic study have been presented in part at the International Congress of Clinical Pathology of September 7, 1954, Washington, D. C., and will be included in a later report. Abstracts of part of the chemical aspects appear in the proceedings of the meeting of the American Association for Cancer Research (*18, 19*).

[7] Designed with Mr. Frederick Highhouse, Instruments Section, National Institutes of Health.

specimens of urines that were obtained for the separate experiments appear in the legends of text-figures 1 and 2.

G. *ACTH administration.*—Animals given ACTH received 10 U of Armour's lyophylized adrenocorticotrophic hormone by intraperitoneal injection immediately before they were placed in a urine-collection cage. When multiple injections were given, these were spaced no closer than 48 hours.

II. Chemical

A. *Quantitative determination of urinary corticosteroids.*—The quantitative determination of the steroids was done as previously reported in detail (*14*), and only a brief description is given here. The urines were extracted with ethyl acetate and the extracts chromatographed in the chloroform-formamide system for 15 to 17 hours. Three blue-tetrazolium reducing zones previously designated as zone I, zone II, zone VII (*14*) were regularly found. These 3 zones, respectively, contain *6β-hydroxycortisol*, *2α-hydroxycortisol*, and *cortisol* as their major components (*13,14*). The steroids in zones I and II were further purified for quantitative determination by acetylation and by rechromatography in the toluene-propylene glycol system. The major reducing zones (*14*) were then determined by ultraviolet spectrophotometry. The third reducing zone, previously shown to contain *cortisol* (zone VII) was rechromatographed directly in the toluene-propylene glycol system for 17 hours and determined by ultraviolet spectrophotometry.

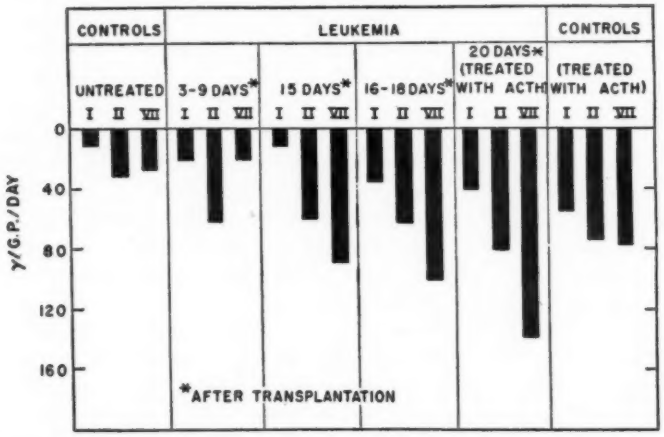
B. *Steroid isolation and identification.*—The steroids were isolated by crystallization of the combined corresponding chromatographic zones. The isolated steroids were identified by infrared spectrometry and by their characteristic spectra in sulfuric acid. Their melting points (uncorrected) were determined with the Kofler micro-hot-stage melting point apparatus. The infrared spectra were recorded on solid films deposited on sodium chloride plates in the Perkin-Elmer 12 C model unless otherwise stated. The absorption spectra in sulfuric acid were obtained with the Cary 11 MS spectrophotometer immediately (within 0.5 minutes) and 2 hours after dissolving the corticosteroids in sulfuric acid, and compared with authentic samples of *cortisol*, *6β-hydroxycortisol diacetate*, and *2α-hydroxycortisol diacetate* (*12–15*).

Results

A. Excretion of Free Reducing Corticosteroids in Guinea Pigs with Leukemia

The excretion of the 3 reducing corticosteroids—*6β-hydroxycortisol*, *2α-hydroxycortisol*, and *cortisol*—in control and in leukemic strain 2 guinea pigs before and after the administration of ACTH is presented in text-figure 1. After the transplantation of leukemia L2B (text-fig. 1), there was a steady increase in the concentration of all the 3 urinary steroids studied. Noteworthy is the elevation of *2α-hydroxycortisol* early in the

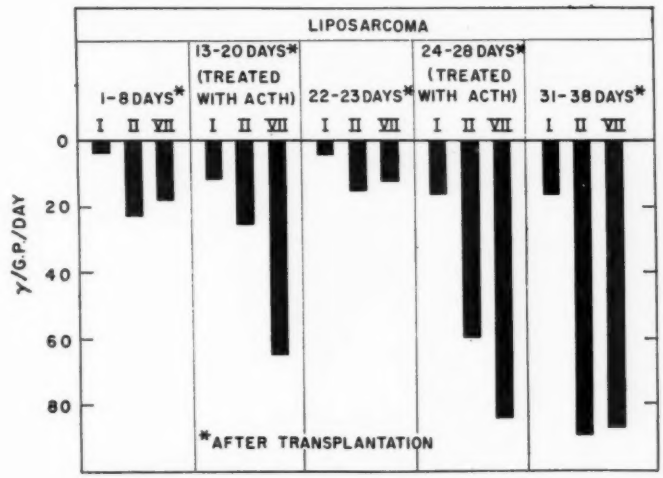
course of the disease (3–9 days after transplantation) when the concentration of urinary *cortisol* was still at the normal level. During the late stages of the disease (16–18 days after transplantation), *cortisol* was excreted in the highest concentration. The excretion of *6β-hydroxycortisol* did not change consistently during the development of the disease and reached high levels only late in the disease. Sixteen to 18 days after transplantation, the total excretion of the 3 steroids exceeded by 180 percent the excretion of untreated control animals and equaled that of control animals that had received 10 U of ACTH. It is remarkable that following the administration of ACTH to terminal leukemic animals (20 days after transplantation) there was a further increase in excretion of only 28 percent (text-fig. 1).



TEXT-FIGURE 1.—Corticosteroid excretion in strain 2 guinea pigs. The values are expressed as γ *cortisol*. The numbers of individual urinary specimens used in the separate experiments are given below:
(*a*) untreated controls, 100; (*b*) 3 to 9 days after transplantation, 56; (*c*) 15 days after transplantation, 28; (*d*) 16 to 18 days after transplantation, 60; (*e*) treated with ACTH on the 20th day after transplantation, 16; (*f*) controls (treated with ACTH), 20. I, II, and VII represent *6β-hydroxycortisol*, *2α-hydroxycortisol*, and *cortisol*, respectively.

B. Excretion of Free Reducing Corticosteroids in Guinea Pigs with Liposarcoma

The excretion values of the 3 urinary corticosteroids during the course of liposarcoma D4888 are shown in text-figure 2. It is evident (text-figs. 1 and 2) that early after transplantation, the corticosteroid-excretion response to the presence of this tumor was much less than after transplantation of leukemia L2B. In fact, the excretion of the 3 steroids, 1 to 8 days after liposarcoma transplantation, approximated the low values of the untreated control animals (text-fig. 1). The urinary excretion remained low even 22 to 23 days after transplantation, but increased to



TEXT-FIGURE 2.—Corticosteroid excretion in strain 2 guinea pigs. The values are expressed as γ *cortisol*. The numbers of individual urinary specimens used in the separate experiments are given below:
(*a*) 1 to 8 days after transplantation, 64; (*b*) treated with ACTH during the 13th to 20th day after transplantation, 64; (*c*) 22 to 23 days after transplantation, 30; (*d*) treated with ACTH during the 24th to 28th day after transplantation, 39; and (*e*) 31 to 38 days after transplantation, 24.

high levels during the terminal stages (31–38 days), when it approximated that of animals in the late stages of leukemia (16–18 days). The rise in steroid excretion following ACTH administration early in the course of liposarcoma (13–20 days) was smaller than that observed in control animals given ACTH. The rise following ACTH administered during the 24- to 28-day period after transplantation approximated that of control animals given ACTH. It is remarkable that the excretion values of untreated animals in the terminal stages (31–38 days) slightly exceeded that of animals given ACTH 24 to 28 days after transplantation.

C. Steroid Isolation and Identification

6β-Hydroxycortisol.—This steroid was isolated (as the diacetate) from the combined eluates obtained from the urine of the animals with liposarcoma. Two crystallizations from methanol gave colorless needles melting at 110 to 118° C. The infrared spectrum of this material agreed with that of *6β-hydroxycortisol diacetate monohydrate* described elsewhere (*13,14*) and contained the following bands: hydroxyl at 3650, acetate carbonyl at 1738, C-20 carbonyl at 1720, C-3 conjugated carbonyl at 1670, and acetate at 1240 cm.$^{-1}$. The spectrum in sulfuric acid was identical with that of *6β-hydroxycortisol diacetate monohydrate*. After dissolving in sulfuric, a transient violet color was seen. The following bands were found immediately after solution of the steroid in H_2SO_4: 387 (0.37), 280 (0.70), and 239 (0.93) mμ; after 2 hours the absorption maxima were

located at 475 (0.36), 387 (0.42), 340 (0.71), 280 (0.62) (band at 280 was considerably flattened), and 239 (0.91) mμ. The values in parentheses are the optical densities for *ca.* 50 γ of steroid in 3 ml.

2α-Hydroxycortisol.—This $C_{21}O_6$ steroid was isolated as the diacetate from the combined fractions of the reducing zones obtained following the chromatography of the acetylated zone II material from the animals with liposarcoma. The material was crystallized twice from methanol to afford prisms which melted at 218 to 223° (some larger crystals melted at 226 to 231°) and was identified as *2α-hydroxycortisol diacetate* by infrared spectrophotometry (*13–15*). The following bands were observed: hydroxyl at 3650, acetate carbonyl at 1740, C-20 carbonyl at 1720, C-3 conjugated carbonyl at 1700, δ 4-double bond at 1625, and acetate at 1245 to 1225 cm.$^{-1}$. In sulfuric acid a green fluorescence was observed, and the spectrum exhibited the following absorption maxima immediately after dissolving in H_2SO_4: 500 (1.04), 380 (0.43), 292 (1.79), and 240 (1.57) mμ. Two hours after dissolving in acid the following bands were obtained: 500 (0.77) (broadened), 389 (0.62), 330 (0.92) (shoulder), 291 (1.55), and 240 (1.68) mμ.[8]

Cortisol.—The fractions containing *cortisol* were crystallized from acetone and identified by infrared spectrophotometry. The following bands were recorded (KBr window): hydroxyl at 3367, C-20 carbonyl at 1700, C-3 conjugated carbonyl at 1645, and δ 4-double bond at 1607 cm.$^{-1}$.

Discussion

This study on acute leukemia and on liposarcoma in guinea pigs clearly demonstrates a marked increase in the concentration of 3 urinary corticosteroids—*cortisol*, *6β-hydroxycortisol*, and *2α-hydroxycortisol*—during the very late stages of these malignant neoplastic diseases. Assuming that corticosteroid-excretion studies are a reliable reflection of adrenocortical activity, it seems that the guinea pig responds to the general stress associated with these neoplastic diseases by increased adrenocortical activity.

The steeper and faster rise in the concentration of the 3 urinary corticosteroids during the development of the leukemia, as compared with the flatter and slower rise after the transplantation of the liposarcoma, evokes interest. The more rapid response in leukemia may be associated with the more acute character of this disease which causes an earlier death than does liposarcoma. It may also be related to the regular observation of leukemia-cell infiltration in the brain and adrenal glands in contrast to the absence of intracranial or adrenal metastases in the animals with liposarcoma. The relatively minor effect of ACTH on the total excretion of these 3 corticosteroids in the very late stages of these 2 neoplastic diseases is probably due to the inability of ACTH to stimulate further an adrenal cortex that is already nearly maximally stimulated. The histo-

[8] The band at 280 mμ was of lower intensity than the corresponding band of Steroid IIa [now identified as *2α-hydroxycortisol* (*15*)] described previously (*15*). Since in subsequent work on this steroid, obtained from urines of untreated as well as of *cortisol*-treated guinea pigs and by synthesis (*15*), sulfuric acid spectra identical with those reported here were obtained, the relatively intense peak around 389 mμ in the original samples of Steroid IIa was probably due to a contaminant present in amounts below the limits of detection by infrared spectrophotometry.

logic evidence of enlarged adrenal glands, depleted of sudanophilic material in these animals with leukemia and liposarcoma, supports this explanation.

The 3 steroids have now been separately determined and identified by means of infrared spectrophotometry, spectra in sulfuric acid, and melting points in the inbred strain 2 guinea pig. In previous studies these 3 steroids were found in noninbred Hartley guinea pigs (*14*).

There are certain pattern differences in the urinary excretion of the 3 corticosteroids in animals terminally ill with leukemia and liposarcoma (text-figs. 1 and 2). However, further studies are necessary in order to determine the significance of these pattern differences. The fact that *6β-hydroxycortisol* (*20,21*) and *cortisol* (*22*) have also been isolated from the urine of man suggests a certain similarity between the biosynthetic mechanisms operating in these 2 species, and it is hoped that the results of the present study may have a bearing on the problem of adrenocortical response to cancer in man (*23*).[9]

Summary

The effect of 2 transplantable tumors (leukemia and liposarcoma) on the excretion of urinary corticosteroids was studied in strain 2 inbred guinea pigs.

In animals with leukemia L2B there was a progressive increase in the concentration of urinary *cortisol*, *2α-hydroxycortisol*, and *6β-hydroxycortisol*. Nearly a threefold increase in the excretion of these 3 steroids in the late stages of these 2 malignant neoplastic diseases was observed. The high excretion in the latter stages approximates that observed following the intraperitoneal administration of 10 U of ACTH to control non-tumor-bearing animals. After the administration of ACTH to animals in the late stages of these malignant neoplastic diseases, the excretion of corticosteroids differed little from that of similar tumor-bearing animals not treated with ACTH.

These 3 corticosteroids have been isolated in crystalline form from pooled urines of normal and tumor-bearing animals, and have been identified as *cortisol*, *2α-hydroxycortisol*, and *6β-hydroxycortisol* by infrared spectrometry, melting points, and by their absorption spectra in sulfuric acid.

Bibliography

(*1*) Dobriner, K., and Lieberman, S.: The metabolism of steroid hormones in humans. *In* Symposium on Steroid Hormones (Gordon, E. S., ed.). Madison, Univ. of Wisconsin Press, 1950, pp. 4–58.

(*2*) Dobriner, K., Kappas, A., and Gallagher, T. F.: Studies in steroid metabolism. XXVI. Steroid isolation studies in human leukemia. J. Clin. Invest. 33: 1481–1486, 1954.

(*3*) Dorfman, R. I., and Ungar, F.: The Metabolism of Steroid Hormones. Minneapolis, Burgess Pub. Co., 1953, 170 pp.

(*4*) Mason, H. L.: Urinary steroids in adrenal disease and metabolism of adrenal hormones. Recent Progress in Hormone Res. 3: 103–123, 1948.

[9] A 6β-hydroxylated product of *cortisol* present in the urine of man and of guinea pigs may be of additional interest in view of the findings of others of the effect of 6β-hydroxylation on the growth properties of some other steroids. Thus, 6β-hydroxylation bestows hormone inhibitory action on an androgen (*3*) and on certain estrogen (*24*) and progesterone (*25,26*) derivatives, makes deoxycorticosterone glycogenic (*3*), and converts certain cholesterol derivatives to carcinogens (*27*).

(5) HANLON, D. G., MASON, H. L., and STICKNEY, J. M.: The urinary excretion of 17-ketosteroids and corticosteroids in leucemia; the effect of aminopterin (4-amino pteroylglutamic acid) on the excretion of corticosteroids in acute leucemia. J. Lab. & Clin. Med. 36: 877–886, 1950.

(6) ENGEL, L. L.: Steroid metabolism in cancer. *In* The Physiopathology of Cancer (Homburger, F., and Fishman, W. H., eds.). New York, Hoeber-Harper, 1953, pp. 687–714.

(7) CONGDON, C. C., and LORENZ, E.: Leukemia in guinea-pigs. Am. J. Path. 30: 337–359, 1954.

(8) HESTON, W. E., and DERINGER, M. K.: Induction of pulmonary tumors in guinea pigs by intravenous injection of methylcholanthrene and dibenzanthracene. J. Nat. Cancer Inst. 13: 705–717, 1952.

(9) NATIONAL RESEARCH COUNCIL: Handbook of Laboratory Animals. Washington, D. C., National Academy of Sciences, Publ. 317, 1954.

(10) ZONDEK, B., and BURSTEIN, S.: The relationship of corticoid excretion to ovarian hormones in the guinea pig. Endocrinology 50: 419–428, 1952.

(11) BURSTEIN, S.: The guinea pig as a laboratory animal for corticoid excretion studies: Modification of the formaldehydegenic method. Endocrinology 50: 412–418, 1952.

(12) BURSTEIN, S., and DORFMAN, R. I.: Hydrocortisone in normal guinea pig urine: isolation and quantitative determination. J. Biol. Chem. 206: 607–612, 1954.

(13) ———: Steroid metabolism in guinea pigs. I. Metabolism of cortisol in vivo. J. Biol. Chem. 213: 581–595, 1955.

(14) BURSTEIN, S., DORFMAN, R. I., and NADEL, E. M.: Corticosteroids in the urine of normal and scorbutic guinea pigs: isolation and quantitative determination. J. Biol. Chem. 213: 597–608, 1955.

(15) BURSTEIN, S.: 2α-Hydroxylation of cortisol in the guinea pig. J. Am. Chem. Soc. 78: 1769–1770, 1956.

(16) NADEL, E. M., and SCHNEIDER, J. J.: Increased excretion of formaldehydegenic ("FG") substances in the urine of scorbutic guinea pigs. (Abstract.) J. Clin. Endocrinol. 11: 791–792, 1951.

(17) ———: Excretion of formaldehydegenic (FG) substances by normal and scorbutic guinea pigs. Endocrinology 51: 5–11, 1952.

(18) NADEL, E. M., BURSTEIN, S., and DORFMAN, R. I.: Urinary excretion of cortisol, 6β-hydroxycortisol, and an unidentified steroid (Steroid IIa) by guinea pigs with leukemia or with liposarcoma. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 37, 1955.

(19) ———: Effect of neoplastic disease and stress on specific urinary corticosteroids in guinea pigs. (Abstract.) Clin. Res. Proc. 3: 42–3, 1955.

(20) BURSTEIN, S., DORFMAN, R. I., and NADEL, E. M.: 6β-Hydroxycortisol, a new steroid in human urine. Arch. Biochem. 53: 307–308, 1954.

(21) NADEL, E. M., BURSTEIN, S., and DORFMAN, R. I.: Isolation of polar reducing corticosteroids from human urine. Arch. Biochem. 61: 144–149, 1956.

(22) SCHNEIDER, J. J.: Studies on the excretion of adrenocortical compounds. I. Isolation of 17-hydroxy-11-dehydrocorticosterone and other compounds from the urine of normal males. J. Biol. Chem. 183: 365–376, 1950.

(23) NADEL, E. M., BURSTEIN, S., YOUNG, B. G., HOLLAND, J., CRAMER, L. M., SMITH, R. R., and FREI, E.: Free (unconjugated) urinary polar corticosteroids in neoplastic disease. (Abstract.) Clin. Res. Proc. 4: 35, 1956.

(24) HUGGINS, C., and JENSEN, E. V.: The depression of estrone-induced uterine growth by phenolic estrogens with oxygenated functions at positions 6 or 16: the impeded estrogens. J. Exper. Med. 102: 335–346, 1955.

(25) MURRAY, H. C., and PETERSON, D. H.: U. S. Patent #2,602,769, July 8, 1952; U. S. Patent #2,672,466, March 16, 1954.

(26) OGILVIE, O., and HANZE, A. R.: U. S. Patent #2,671,091, March 2, 1954.

(27) FIESER, L. F., GREENE, T. W., BISCHOFF, F., LOPEZ, G., and RUPP, J. J.: A carcinogenic oxidation product of cholesterol. J. Am. Chem. Soc. 77: 3928–3929, 1955.

Effect of the Virus of Lymphocytic Choriomeningitis on the Course of Leukemia in Guinea Pigs and Mice [1, 2]

ELI M. NADEL *and* VICTOR H. HAAS,[3] *National Cancer Institute and National Institute of Allergy and Infectious Diseases,*[4] *Bethesda, Maryland*

Introduction

Virus-like contaminants of 4 transmissible tumors in mice have been reported. Thus, "passenger contaminants" have accompanied mammary adenocarcinoma E0771 (*2*), lymphosarcoma T86157 (*3*), line I leukemia (*4,5*) and one subline of acute leukemia L1210 (*6*). Cytologic alterations in the tumor cells were said to accompany "virus" infection of the adenocarcinoma and of the lymphosarcoma, while inhibition of tumor growth occurred when either the line I or the L1210 leukemias were "infected" by their particular virus-like contaminant.

The alteration of the growth rate of the tumor and the survival time of the host with the line I or the L1210 leukemia, when contaminated by an agent, invited further study. In the report on line I it was shown that when the same dosage of cells was used for transplantation the survival of mice inoculated with "contaminant-infected" leukemia cells was longer than that of animals inoculated with "noninfected" tumor cells. Also, when line I leukemia was freed of the contaminant, the survival time of the mice was reduced (*5*). It was later demonstrated that the contaminant of line I leukemia had a close immunologic relationship to the virus of lymphocytic choriomeningitis (LCM) (*7*). Although distinct from LCM virus strains then available, it was believed that the virus infecting line I, which had appeared during 10 years of tumor passage (*7*), was a variant of the LCM virus. Whether the virus-like contaminant of leukemia L1210 and the visceral lesions in contaminant-carrying mice reported by Law and Dunn (*6*) were related to LCM virus or to the line I virus can only be speculated upon now, for immunologic tests were not undertaken when the L1210 contaminant was available for study. The morphologic lesions found in mice with the L1210 contaminant were described in detail. They were not identical with the

[1] Received for publication May 21, 1956.

[2] Presented in part as an abstract before the American Society for Experimental Pathology at its 40th annual meeting in San Francisco, California, April 1955 (*1*).

[3] We wish to thank Mr. Bobby G. Young and Mr. Arthur C. Hilgar for their technical assistance, and Mr. Roskey Jennings for laboratory assistance.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

lesions described for line I, but they had certain features resembling LCM infection.

In view of these suggestive findings we have tested 3 known strains (WCC, WCP, WCF) of LCM virus (*8*) for their effect on the course of the transplantable leukemia L2B in inbred guinea pigs and on the L1210 leukemia in mice. In a few experiments, 3 other viruses (St. Louis encephalitis, influenza, and yellow fever) were used in conjunction with LCM in guinea pigs. These studies, reported here, indicate that the LCM virus had a certain inhibitory effect upon the growth of leukemia L2B in guinea pigs.

Materials and Methods

Experimental Design

One hundred and seventy-one strain 2 guinea pigs were divided into 8 control and 16 experimental groups. Those in any series were of approximately the same age, sex, and weight. The experimental design, comprising 8 series, is shown in table 1. Live virus was injected subcutaneously or intraperitoneally at periods varying from 32 days prior to transplantation of the leukemia to 30 days after transplantation. In calculating survival, the date of transplantation of the leukemia was taken as day 0. The leukemia was transplanted intraperitoneally in series 7 and subcutaneously in all others.

Earlier work (*9*) indicated that the original strains of LCM did not produce as severe infections in the guinea pig as in the mouse, and preliminary studies with the WCC strain of the virus confirmed this. LCM-infected guinea pigs became ill as early as the 3rd day after subcutaneous injection, and remained ill for as long as 11 days as judged by ruffled fur, increased body temperature, and labored breathing. A second injection of LCM virus did not elicit a repetition of this clinical course, and such animals were designated as immune. (Actually they were hyperimmunized by the second injection.) Animals thus immunized and others receiving only dead virus were included as controls in series 5, while all other series contained only untreated controls. In addition to the 2 strains of LCM virus (WCC and WCP) used in the 8 series, St. Louis encephalitis (SLE), influenza PR-8 (I-PR-8), and yellow fever 17D (YF-17D) viruses were administered to animals in series 5 and 6. In the 8th series, leukemic animals receiving LCM virus alone were contrasted with leukemic controls and with those receiving both LCM virus and ACTH.

In 3 related experimental series using 113 mice, LCM virus was tested for its ability to alter the course of L1210 leukemia (table 2). Virus was injected intraperitoneally into uninfected mice, as well as into mice exposed to or infected with LCM *in utero* as described in the section on virus preparation, either 2 days before the transplantation of the leukemia or on the day after.

Every guinea pig was given a *post-mortem* examination, including the central nervous system. Body weights, spleen, tumor, and adrenal sizes were recorded and representative tissue sections were removed, fixed in

TABLE 1.—*Effect of LCM* virus on survival time and spleen and tumor size of guinea pigs with leukemia L2B/N†*

Series	Number of animals	Treatment	Survival (*days*) Mean ± S.E.	Effect of treatment on survival (*days*)	Spleen vol. (*cc.*) Mean ± S.E.	Tumor vol. (*cc.*) Mean ± S.E.
1	4	None	16.0 0.0		8.4 1.4	1.8 0.5
	6	LCM day (−2)	26.5 1.2	Plus 10.5	4.9 2.1.	10.7 2.5
	6	LCM day (+2)	29.8 2.2	Plus 13.8	6.9 1.7	9.4 3.9
2	7	None	24.1 3.3		5.8 3.2	11.2 5.6
	8	LCM day (+4)	58.2 6.4	Plus 34.1	1.9 0.9	5.2 2.1
	5	LCM day (−28) or (−30)‡	20.4 2.0	Minus 3.7	12.6 3.4	7.9 1.6
3	10	None	16.8 0.6		16.7 1.9	6.7 0.9
	5	LCM day (+7)	26.4 2.8	Plus 9.6	5.6 2.6	13.1 6.3
	10	LCM day (+3)	25.6 2.3	Plus 8.8	3.2 1.3	9.8 4.3
4	10	None	21.0 1.8		18.9 2.6	6.6 3.2
	5	LCM day (+11)	21.6 2.0	Plus 0.6	4.6 1.4	4.3 0.6
	6	LCM day (+12)	20.8 3.7	Minus 0.2	12.1 3.4	5.8 2.8
5	3	None	17.0 0.0		Not measured	Not measured
	3	Immunized; then dead LCM day (+3)	18.3 0.3	Plus 1.3	Not measured	Not measured
	4	Dead LCM day (+3)	16.5 0.8	Minus 0.5	9.7 4.4	1.5 0.5
	5	LCM day (+3)	31.8 3.6	Plus 14.8	1.8 2.3	11.6 3.3
	5	LCM day (+3)§	37.4 0.6	Plus 20.4	1.2 0.3	28.3 8.6
	10	Immunized; then LCM day (+3)	21.9 2.0	Plus 4.9	13.4 1.3	6.9 2.8
6	6	None	19.2 3.2		1.3 0.4	11.4 7.8
	5	LCM day (+4)	34.2 8.0	Plus 15.0	4.1 0.8	7.3 3.1
	6	LCM day (+4)‖ and (7)	32.0 5.3	Plus 12.8	3.1 0.9	14.8 5.6
	9	LCM day (+4)¶	31.1 4.9	Plus 11.9	5.7 1.4	9.9 1.9
7	4	None	17.0 0.0		11.7 1.6	....
	10	LCM day (+3)	23.5 0.6	Plus 6.5	3.6 0.8	....
8	5	None	16.5 0.2		14.8 1.8	3.7 0.9
	5	LCM day (+5)	24.2 3.4	Plus 8.3	8.5 3.5	17.5 6.1
	5	LCM day (+5) plus daily ACTH	19.4 2.6	Plus 2.9	9.8 4.9	16.2 9.1

* LCM virus (WCC strain) used in all series and injected subcutaneously, 0.1 cc. of a 10^{-3} dilution of infected mouse brain; dead virus was prepared by heating an infected mouse-brain suspension for 45 minutes at 56° C. (series 5); immunized animals were those infected initially with LCM virus and injected again with live virus 2 weeks later.

† Leukemia L2B/N transplanted on day 0 into right axillary space in series 1 to 6 and 8, and intraperitoneally in series 7; brei dilutions of tumor and spleen in Locke's solution were used as follows: *series 1–5*, ½ cc. of a 1:2 brei; *series 4–6*, ¼ cc. of a 1:1 brei; *series 7–8*, ¼ cc. of a 1:2 brei.

‡ Of 5 guinea pigs, 2 were infected 28 days and 3 were infected 30 days before transplantation of leukemia.

§ LCM virus injected 3 days after and SLE virus injected 30 days after transplantation of leukemia.

‖ LCM virus (WCC strain) injected 4 days after and LCM virus (WCP strain) injected 7 days after transplantation of leukemia.

¶ LCM virus injected 4 days after, YF-17D injected 9 days after, I-PR-8 injected 14 days after, and SLE injected 20 days after transplantation.

TABLE 2.—*Effect of LCM virus on survival of mice with leukemia L1210**

Series	Mice	Number	L1210	LCM	Survival (*days*) Mean ± S. E.
1	S(WCF)DF_1	6	+	nil	11. 6 0. 8
	S(WCF)DF_1	6	+	nil	11. 0 0. 7
	BDF_1	5	+	nil	14. 6 1. 2
	BDF_1	6	+	nil	14. 8 0. 8
	CDF_1	5	+	nil	15. 4 0. 7
2	BDF_1	5	+	Day (+1)	6. 4 0. 2
	BDF_1	5	+	nil	6. 0 0. 2
	SDF_1	4	+	Day (+1)	6. 3 0. 2
	SDF_1	3	+	nil	6. 0 0. 2
	S(WCF)DF_1	5	+	Day (+1)	6. 4 0. 2
	S(WCF)DF_1	5	+	nil	6. 8 0. 2
	BDF_1	5	+	Day (+1)	6. 8 0. 2
3	B(EIU)DF_1	5	+	Day (−2)	7. 2 0. 4
	B(EIU)DF_1	5	+	nil	6. 2 0. 2
	B(EIU)DF_1	5	+	Day (−2)	8. 0 0. 4
	B(EIU)DF_1	5	+	nil	6. 0 0. 0
	NIHDF_1	5	+	Day (−2)	7. 0 0. 4
	NIHDF_1	3	+	nil	6. 0 0. 0
	S(WCF)DF_1	5	+	Day (−2)	5. 8 0. 2
	S(WCF)DF_1	5	+	Day (−2)	5. 8 0. 2
	S(WCF)DF_1	5	+	nil	5. 6 0. 2
	S(WCF)DF_1	5	+	nil	5. 6 0. 2
	BDF_1	5	+	Day (−2)	7. 6 0. 2

*Leukemia L1210 transplanted on day 0 into right axillary space using 0.1 cc. of a 1 : 2 brei of tumor and spleen in Locke's solution in each series. Leukemia L1210 S (*ss*) used in series 2 and 3, leukemia L1210 R (*rr*) used in series 1.

LCM virus (WCC strain) injected subcutaneously using 0.1 cc. of a 10^{-3} dilution of infected brain tissue in series 2 and 3; no virus injected in series 1.

S(WCF)DF_1 are hybrids infected *in utero* with LCM virus (WCF strain) present naturally in infected S(WCF) mothers; virus was recovered from the blood of these hybrids.

B(EIU)DF_1 are hybrids exposed *in utero* to LCM virus by injection of the WCF strain into pregnant C57BL/6 mice; virus could not be demonstrated in these hybrids born of virus-infected mothers.

10 percent formalin, and prepared for histologic study. Tumor and spleen volumes were measured by the method of Bunting (*10*). Measurements on dissected spleens and tumors were made in 3 dimensions using a millimeter caliper. The formula used was

$$\text{volume} = \frac{4}{3}\pi \times \frac{(\text{length})}{2} \times \frac{(\text{depth})}{2} \times \frac{(\text{width})}{2}.$$

In the studies on mice, survival data were collected and clinical impressions recorded. A few representative tumors and tissues from untreated leukemic and LCM-treated leukemic mice were fixed in 10 percent formalin and prepared for histologic study.

Viruses

LCM.—One strain, designated WCC, was employed as a suspension of the brains of mice from a series in which infection is maintained by intracerebral passage (*8*). Another strain, designated WCP, was used in the form of a suspension of spleen, kidney, and liver of mice from a series

infected by intraperitoneal passage (*8*). Tissues were ground by hand with mortar and pestle. Dilutions (1:1000) were made with buffered physiologic saline solution and injected subcutaneously without prior centrifugation. Both strains of virus are used routinely in the laboratory for other investigations, and their viability is thus under constant observation. No standardized preparation with known end points is maintained, but experience has been that the tissues as used are uniformly infective to mice at least 2 log dilutions beyond those used here. The effect of these strains of virus on guinea pigs was not studied previous to the current investigations.

A third source of LCM virus was a group of mice infected by *in-utero* transmission (*8*). When this strain, designated WCF, was injected, it was employed as whole blood diluted 1:10 in buffered physiologic saline.

SLE.—This virus, derived from the original (Freeman) strain (*11*), was prepared from infected mouse brains of an intracerebral-passage series and injected (intraperitoneally) in a 1:1000 dilution in buffered saline.

I-PR-8.—This virus [7] was obtained as infected allantoic fluid from chick embryos and was given intranasally, diluted 1:50 in saline.

YF-17D.—Commercial yellow fever vaccine [8] was diluted 1:10 with buffered physiologic saline and injected intraperitoneally.

Guinea Pigs

Strain 2 guinea pigs, highly inbred by repeated brother × sister matings, were supplied by the Animal Breeding Section of the Laboratory Aids Branch, National Institutes of Health (*12*). They were housed in 18 × 36″ transparent plastic cages, 3 to 5 in a cage, and fed Purina rabbit chow pellets and cabbage daily. Water was always available. Care was used in changing and in cleaning the cages to prevent any contamination of noninfected animals by the droppings or secretions of infected animals. No difference in survival or in the course of the leukemia in untreated controls maintained in the isolation room was noted in comparison to that of other animals receiving transplants at the same time and maintained in another building. All animals were examined and palpated daily for tumor growths, and any alterations in their clinical course were noted.

Mice

Normal uninfected DBA/2 (D), C57BL/6 (B), NIH BALB/c (C) and (S) mice were supplied by the Animal Breeding Section. Swiss mice naturally infected with LCM *in utero* (WCF) were derived from a colony maintained in the National Institute of Allergy and Infectious Diseases and are designated as S(WCF). Litters of mice exposed *in utero* to LCM by inoculation of the mothers with the WCC late in pregnancy are desig-

[7] We are indebted to Dr. Bernice Eddy Wooley for the influenza virus.
[8] National Drug Co. We are indebted to Dr. John Hornibrook for this material.

nated as exposed *in utero* (EIU). Viremia was not demonstrated in these (EIU) litters. The following hybrid mice were used:

(1) (BALB/c × DBA/2)F_1 or CDF_1
(2) (C57BL/6 × DBA/2)F_1 or SDF_1
(3) (Swiss × DBA/2)F_1 or $NIHDF_1$
(4) (NIH × DBA/2)F_1 or $NIHDF_1$
(5) [C57BL/6 (EIU) × DBA/2]F_1 or $B(EIU)DF_1$
(6) [Swiss (WCF) × DBA/2]F_1 or $S(WCF)DF_1$

Animals weighed between 18 and 22 grams when L1210 was transplanted.

Transplantable Leukemias

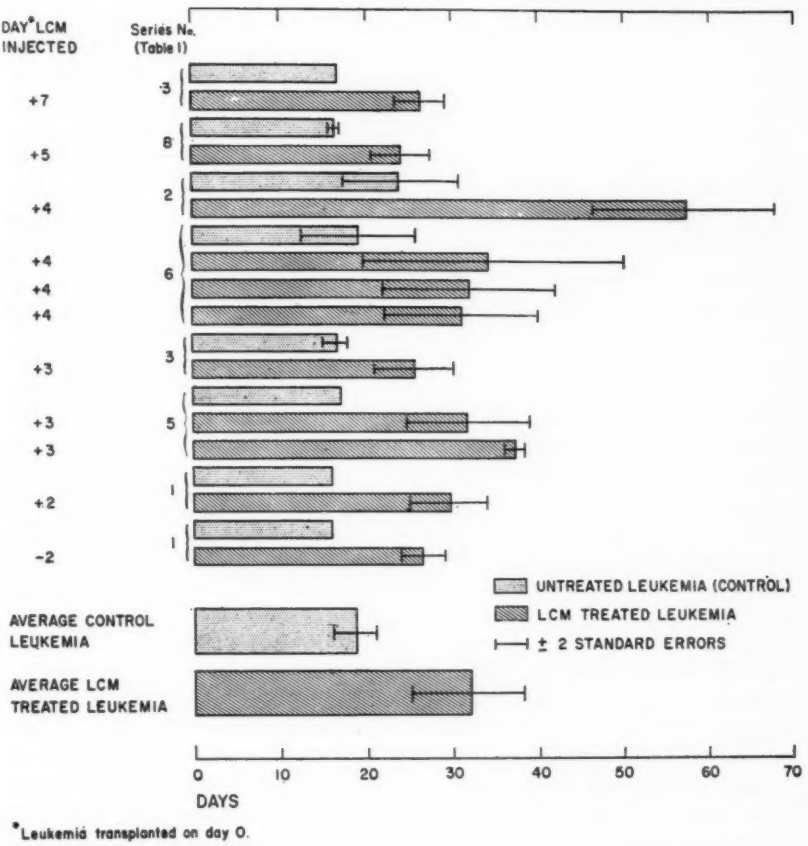
An acute leukemia, L2B/N, maintained for over 55 continuous passages, was used. The tumor had its origin in a lymphoma that developed in an inbred strain 2 guinea pig exposed daily, over a prolonged time, to low dosage whole-body irradiation (*13*). The original tumor, known as leukemia L2B, was supplied to us in its 22nd transplant generation by the late Dr. Egon Lorenz. It has been maintained in the Laboratory of Pathology, National Cancer Institute, by passages in young newborn, non-inbred and inbred strain 2 animals (*14*) for over 50 transplant generations. After sufficient numbers of inbred animals became available, the tumor was maintained continuously in strain 2 guinea pigs. The duration of life after transplantation of the original tumor received from Dr. Lorenz averaged between 14 and 17 days. At the time of the present experiments the average survival ranged between 16 and 21 days. In all other respects our variant, L2B/N, appears to be similar to the original L2B now maintained by Dr. Julius White, in the Laboratory of Physiology, National Cancer Institute. Leukemia L1210 (*15*) was supplied by Dr. Lloyd Law, Leukemia Studies Section, National Cancer Institute. In our experience, both leukemia L2B and L1210 are universally fatal after either subcutaneous or intraperitoneal injection of tumor tissue obtained from either the localized tumor or the spleen. A brei of tumor cells was prepared by hand grinding tumor tissue in a ground-glass test tube mortar and pestle. Subcutaneous injections were made into the right axillary space in guinea pigs (series 1–6 and 8) and by intraperitoneal injections through the lower right abdominal wall (series 7). Mice received subcutaneous implants into the right axillary space. Various amounts were given, ranging from ¼ cc. to ½ cc., of a 1:2 to a 2:1 brei as indicated in tables 1 and 2. Aseptic techniques were used.

Results

Guinea Pigs

The results of the study in guinea pigs are shown in table 1 and in text-figure 1. The leukemic guinea pigs treated with live LCM virus lived longer than their untreated controls (table 1) if the virus was administered during an interval from 2 days prior to transplantation to as late as 7 days

after transplantation. Injection of live virus before or after this particular time range did not significantly alter the survival time. This is shown graphically in text-figure 1. A maximum increase in survival was noted in animals treated with LCM virus 4 days following transplantation of the leukemia.



TEXT-FIGURE 1.—Effect of LCM virus on survival of guinea pigs with leukemia.

Animals previously immunized with live LCM virus did not survive longer when they were subsequently given LCM virus treatment for leukemia (table 1). Dead LCM virus (heat-killed, 56° C., 45 minutes) was not effective in altering the course of leukemia L2B in guinea pigs. Prolongation of life was noted only when live LCM virus was used in guinea pigs that had no immunity to it. In series 5 a slight increase in survival was noted for the combination of LCM and SLE virus over that of leukemic animals given LCM virus alone. Successive injection with 4 viruses—LCM, YF-17D, I-PR-8, and SLE—given at 4, 9, 14, and 20

days, respectively, after the transplantation of leukemia L2B produced no greater effect in prolonging life than did the injection of LCM virus alone (series 6).

Comparison of the spleen and local axillary-tumor volumes in LCM-treated leukemic guinea pigs and their untreated leukemic controls (table 1) suggests that LCM infection has some effect in localizing the tumor growth. Leukemic animals (with axillary transplants) treated with LCM virus had larger local tumors in all but series 2 and smaller spleens than the controls in all but series 6. A more positive effect of LCM virus on spleen size was seen in the animals with intraperitoneal transplants (series 7) and in series 3. In these, the leukemic spleens of virus-treated animals were less than one third the size of the spleens of untreated leukemic controls.

Another difference was detected only in 8 virus-treated leukemic animals that survived more than 28 days. This was the presence of a localized infiltration of tumor in the fundus oculi. Retro-orbital growth of tumor tissue caused proptosis and subsequent ulceration and inflammation of the cornea.

Mice

In 3 series, LCM-virus infection did not significantly increase survival of hybrid mice bearing the L1210 transplantable leukemia (table 2). In series 1, mice infected *in utero* with WCF strain [S(WCF)DF_1] survived for a shorter period than did other hybrids not infected *in utero* (BDF_1 and CDF_1). In series 2 and 3, there was no significant difference in survival whether or not the leukemic mice had received an injection of virus (WCC strain). In series 3, however, leukemic mice infected *in utero* [S(WCF)DF_1] and subsequently injected with the WCC strain, survived for a significantly shorter time than other hybrids.

Death in mice could be attributed to either LCM virus or leukemia, or to both. In series 1, where mice were not experimentally infected with virus, it was believed that the deaths were leukemic deaths because of the survival beyond the usual expected time for LCM-attributable deaths in mice (*8*) and because of the large axillary tumors. In series 2 and 3, however, the tumor masses were smaller in virus-treated mice, as judged by palpation. It is uncertain whether the decrease in tumor size and the short survivals could be attributed to virus infection. Were a DBA/2 F_1 hybrid found that would be relatively more resistant to the virus of LCM, it might be more suitable for a study of the effect of LCM on the course of leukemia L1210 in mice (*16*). Alternatively, the use of a strain of LCM less virulent for mice or an LCM strain adapted to tumor tissue might permit such a study (*17*).

Discussion

Increased survival of tumor-bearing guinea pigs experimentally infected with LCM virus has been observed in 16 individual experiments utilizing 171 animals. These results were obtained only when virus was inoculated

from 2 days before to 7 days after transplantation of the leukemia, and only after subcutaneous or intraperitoneal injection of live virus into non-immunized animals. Dead virus did not have any effect on the survival time. Normal animals given dead virus, leukemia, and live virus in that sequence showed an increased survival over that of untreated leukemic controls. It was not necessary to modify the virus by repeated passage in tumor cells to obtain the effect of prolonged survival (*17*). The reasons for this increased survival after LCM injection warrant further study, since none of the agents that alter murine or human acute leukemia—such as cortisone and ACTH, given separately or together, or amethopterin—when given in comparable doses, will alter the course of leukemia L2B/N in guinea pigs. The other viruses used in the present experiments likewise had no effect on the course of the leukemia. These facts suggest that the inhibitory effect of LCM virus on leukemia in guinea pigs may be due to direct interference by the virus on the growth of leukemia cells and not to a nonspecific effect of stress. In support of this are the following additional facts: (*a*) A particular time relationship (−2 to +7 days) between infection and transplantation was necessary to produce the sparing effect. This period was apparently related to the time required by the virus to multiply, and was the period during which some clinical manifestations of LCM-virus infection were observed in the guinea pigs. (*b*) If active immunization against LCM preceded the transplantation of leukemia and injection of the virus, there was no interference with the course of leukemia. (*c*) The administration of ACTH alone or in combination with LCM virus did not increase the survival time. It was therefore not considered likely that the observed sparing effect of LCM virus on survival after leukemia transplantation was the result of a nonspecific effect. (*d*) Others have reported specific tumor-inhibitory properties of many viruses (*18,19*) without significant prolongation of survival (*20,21*).

The retro-orbital growths noted in 8 animals surviving for longer than average periods after LCM treatment of their leukemia, suggested that the increase in survival allowed those leukemia cells that had disseminated to the brain to grow and form local tumors. Study of the effect of neurotropic viruses on cancer in man has shown greater susceptibility of patients with leukemia to develop encephalitis and histopathologic lesions (*22*). This and the inflammatory reaction known to occur with the virus of LCM may have been factors in permitting a more luxuriant localized growth of tumor cells. Transplantation of the retro-orbital tumor into uninfected strain 2 guinea pigs resulted in the death of these animals with leukemia in the expected time for leukemia L2B. Retro-orbital tumors were removed more than 28 days after LCM injection, at which period we have shown the virus was no longer active (table 1, series 2).

No significant effect of LCM virus on survival of mice with leukemia L1210 was noted in the 3 series studied. However, LCM virus (WCC strain) and the L1210 leukemia have overlapping incubation periods. This period might have been too short for beneficial virus-leukemia-host interaction. A sparing effect of the line I contaminant on line I leukemia

was demonstrable only when small numbers of leukemia cells were used in transplantation (*5*). In our study no attempt was made to dilute leukemia L1210. The doses were chosen to given a uniform and rapid killing time.

During the course of virus infection in guinea pigs we believe biologic damage occurs in leukemia cells either by direct injury or, secondarily, from injury to the host, and that some interaction between leukemia cells and virus takes place to account for the increased local tumor growth and decreased spleen size in virus-treated leukemic animals. During virus infection the leukemia cells in the local growth appear to be less capable of extensive invasion, and they proliferate locally for a longer period. On the other hand, leukemia cells not exposed to virus leave the local tumor-inoculation site more quickly and begin to proliferate in the spleen, causing it to increase in size. A parallel situation was reported where smaller spleens were present in leukemic mice infected with line I virus than in leukemic mice free of virus (*3*).

Summary

In 8 series (16 experiments), utilizing 171 guinea pigs, the experimental infection with the virus of lymphocytic choriomeningitis significantly increased the survival and decreased the spleen size of guinea pigs bearing the transplantable leukemia L2B/N. The effectiveness of the virus was limited to a period of injection from 2 days before to 7 days after transplantation of the leukemia. Previously immunized guinea pigs were afforded no protection by live virus. Killed virus afforded no protection against leukemia. During the effective period, 70 LCM-treated leukemic guinea pigs lived 32.5 ± 3.1 days, in contrast to 18.3 ± 1.4 days for 36 untreated leukemic controls. Sequential injection of other viruses (St. Louis encephalitis, influenza, yellow fever) did not add to the sparing effect of LCM virus on the course of leukemia in guinea pigs. In contrast, LCM infection did not significantly increase the survival of mice with leukemia L1210.

References

(*1*) NADEL, E., and HAAS, V.: Inhibitory effect of virus of lymphocytic choriomeningitis on the course of leukemia in guinea pigs. (Abstract.) Fed. Proc. 14: 414–415, 1955.

(*2*) KRITZLER, R. A., MULLIKEN, B., and TURNER, J. C.: A hitherto undescribed virus forming inclusion bodies in the organs and an experimental tumor of C57 mice. Cancer 4: 401–409, 1951.

(*3*) DEBRUYN, W. M.: A lymphopenia-causing agent, probably a virus, found in mice after injection with tumor tissue and with cell-free filtrates of lymphosarcoma T 86157 (MB). Cancer Res. 9: 395–397, 1949.

(*4*) MACDOWELL, E., TAYLOR, M., and BROADFOOT, J.: Mouse leukemia. Carnegie Inst. Wash. Year Book No. 49: 178–181, 1949–1950.

(*5*) TAYLOR, M. J., and MACDOWELL, E. C.: Mouse leukemia XIV. Freeing transplantable line I from a contaminating virus. Cancer Res. 9: 144–149, 1949.

(*6*) LAW, L. W., and DUNN, T. B.: Effects of a filtrable, self-propagating contaminant on a transplantable acute lymphoid leukemia in mice. J. Nat. Cancer Inst. 11: 1037–1055, 1951.

(7) LINDORFER, R. K., and SYVERTON, J. T.: The characterization of an unidentified virus found in association with line I leukemia. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 33–34, 1953.

(8) HAAS, V. H.: Some relationships between lymphocytic choriomeningitis (LCM) virus and mice. J. Infect. Dis. 94: 187–198, 1954.

(9) LILLIE, R. D., and ARMSTRONG, C.: Pathologic reaction to the virus of lymphocytic choriomeningitis in guinea pigs. Pub. Health Rep. 59: 1391–1405, 1944.

(10) BUNTING, H.: Studies of regression in a transplantable tumor in mice. Yale J. Biol. & Med. 13: 513–522, 1941.

(11) MUCKENFUSS, R. S., ARMSTRONG, C., and MCCORDOCK, H. A.: Encephalitis: Studies on experimental transmission. Pub. Health Rep. 48: 1341–1343, 1933.

(12) NATIONAL RESEARCH COUNCIL: Handbook of Laboratory Animals. Washington, D. C., National Academy of Sciences, Publ. 317, 1954.

(13) CONGDON, C. C., and LORENZ, E.: Leukemia in guinea-pigs. Am. J. Path. 30: 337–359, 1954.

(14) NADEL, E. M.: An influence of age on transplantation of leukemia from inbred to non-inbred guinea pigs. (Abstract.) Fed. Proc. 13: 439–440, 1954.

(15) LAW, L. W., DUNN, T. B., BOYLE, P. J., and MILLER, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(16) NADEL, E. M., GREENBERG, J., and COATNEY, G. R.: The effect of malaria (*Plasmodium berghei*) on leukemia L1210 in mice. J. Infect. Dis. 95: 109–116, 1954.

(17) FLANAGAN, A. D., LOVE, R., and TESAR, W.: Propagation of Newcastle disease virus in Ehrlich ascites cells *in vitro* and *in vivo*. Proc. Soc. Exper. Biol. & Med. 90: 82–86, 1955.

(18) MOORE, A. E.: Effects of viruses on tumors. Ann. Rev. Microbiol. 8: 393–410, 1954.

(19) SOUTHAM, C. M., and MOORE, A. E.: Clinical studies of viruses as antineoplastic agents, with particular reference to Egypt 101 virus. Cancer 5: 1025–1034, 1952.

(20) SOUTHAM, C. M., BRONSTEIN, B., and WEBBER, L. F.: Effect of West Nile and Ilhous viruses on mouse leukemias. Cancer Res. 11: 669–675, 1951.

(21) SOUTHAM, C. M., and EPSTEIN, J. D.: The effect of Russian encephalitis and other viruses on mouse leukemia. Cancer Res. 13: 581–586, 1953.

(22) NEWMAN, W., and SOUTHAM, C. M.: Virus treatment in advanced cancer. A pathological study of fifty-seven cases. Cancer 7: 106–118, 1954.

(23) NADEL, E. M., and GREENBERG, J.: Synergistic inhibitory action of a-methopterin and a diaminopyrimidine upon leukemia L 1210 in mice. Cancer Res. 13: 865–868, 1953.

(24) NADEL, E. M., and HILGAR, A. G.: Resistance to quadruple combination chemotherapy in leukemia L1210 in mice. (Abstract.) Am. J. Path. 31: 605, 1955.

Lymphocytic Choriomeningitis Virus in Mouse Neoplasms [1]

SARAH E. STEWART *and* VICTOR H. HAAS, *National Cancer Institute* [2] *and National Institute of Allergy and Infectious Diseases,* [2] *Bethesda, Maryland*

In earlier communications (*1–3*) it was reported that neoplasms of the parotid and adrenal glands could be produced in mice of strains C3H and C3Hf and in (C3Hf × AKR)F_1 hybrids by inoculating them a few hours after birth with cell-free extracts or filtrates prepared from (*a*) leukemic tissues of strain AKR mice, (*b*) leukemias arising in (C3Hf × AKR)F_1 hybrids, or (*c*) transplanted leukemias in mice of strains C3H and AKR. A high incidence of early lymphocytic leukemias was also observed in the hybrid mice inoculated in the same manner.

The cell-free preparations were frequently fatal for a large percentage of the newborn C3H and C3Hf mice inoculated and, to a lesser degree, for newborn (C3Hf × AKR)F_1 hybrids; *i.e.*, the mice died in 9 to 21 days with severe anemia and without tumors. The peripheral blood generally showed a reticulocytosis, anisocytosis, many erythrocytes with Howell-Jolly bodies, and occasional normoblasts. Some mice developed leukocytosis and others, leukopenia; granulocytes predominated in all mice. A preliminary report (*4*) was given on the recovery and propagation of a virus-like agent from the tissues of infant mice that died with these changes after inoculation with cell-free preparations from 3 different transplanted leukemias.

This agent also caused an acute generalized inflammatory disease, affecting most of the organs, in certain adult mice inoculated subcutaneously. In most of these there was atrophy of all lymphoid tissue. Extramedullary myelopoiesis and myelopoietic foci were found in the liver and spleen. Marked erythropoiesis occurred in the spleens of some mice. All these mice died in 12 to 25 days with severe anemia; some had granulocytosis (30,000 to 90,000 white blood cells per $mm.^3$); others had leukopenia. Hypoplastic bone marrow was found in the latter on several occasions.

The present work was directed toward recovering and identifying the agent causing these deaths. It has resulted in recovery of the virus of lymphocytic choriomeningitis (LCM) from several neoplasms. The source of this virus in these particular tumors has not been established.

[1] Received for publication April 10, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

There have been various reports on viruses recovered from mouse leukemias which produce pathologic changes in the hematopoietic system (*5–9*). One reported by Taylor and MacDowell (*5*) has been identified by Lindorfer and Syverton (*10*) as a possible variant of LCM virus; none has been considered etiologically related to tumor formation. Extramedullary myelopoiesis associated with other transplanted mouse tumors has been described (*11–15*), but little has been done to elucidate the cause. Parsons (*16–18*) observed that sarcomas could be induced in mice by inoculation with cell-free filtrates of "mal." sarcoma I, a tumor induced by a derivative of dibenz[*a*,*h*]anthracene. Coincident with the growth of these tumors, the following blood changes were observed: 1) rapid fall in number of lymphocytes; 2) rapid rise in number of polymorphonuclear cells; 3) increase in number of myeloid cells, "blast" forms, and myelocytes; 4) rise in total white blood cells (ranging from 90,000 to 400,000 per $mm.^3$ in rapidly growing tumors); 5) frequent anemia, with occasional erythroblasts. There was extramedullary myeloid metaplasia in the liver and spleen, often with tremendous enlargement of the latter; many myeloid cells were seen. Lymph nodes were atrophied. Upton and Furth (*19*) have described a disease of mice produced by cell-free filtrates and characterized by anemia, leukopenia, splenomegaly, and myelosclerosis.

Materials

Mice

All mice, with the exception of those of strain AKR, were obtained from Dr. G. E. Jay, of the National Institutes of Health, Bethesda, Maryland. The AKR mice were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, and from Dr. L. W. Law, National Cancer Institute, Bethesda, Maryland. The fetuses were obtained from mice bred in the laboratory. All animals were housed in plastic cages and were constantly supplied with Purina laboratory chow and tap water.

Tumors

Three of the leukemias from which a virus-like agent had been recovered previously were described in an earlier report (*2*). A résumé of their origin and characteristics follows:

1) *C3H leukemia #1124.*—This arose 4½ years ago as a generalized leukemia in 1 of 6 C3H male mice, 12 weeks of age, that had been inoculated 3 weeks previously with a homogenate of 6 pooled AKR leukemias adapted to grow in (C × C3H)F_1 hybrid mice (*20*). The leukemia was maintained for 4 years. The adapted tumor grew progressively in the AKR strain of origin, but leukemia #1124 failed to grow on repeated transfers back to AKR mice. It produced a typical lymphoid leukemia in all C3H mice inoculated.

Leukemia #1124 consistently produced hemorrhagic lesions. The peripheral and mesenteric lymph nodes in the original mouse in which

the leukemia developed were greatly enlarged and hemorrhagic. The thymus, liver, and spleen were also greatly enlarged. The peripheral blood consistently showed leukocytosis (40,000 to 400,000 per mm.^{3}). The predominant cell was large and mononucleated, with a large vesicular nucleus, prominent nucleoli, a nuclear membrane, and a narrow ring of basophilic cytoplasm. Using aseptic techniques, all transplants were made subcutaneously into C3H mice 6 to 10 weeks old. Through the first 6 generations, transplants were made every 4 weeks, but thereafter it became necessary to transplant every 2 weeks because of increased "virulence." By the 16th generation the leukemia was killing the mice in 10 to 12 days. The tumor was carried for 114 transplant generations.

2) *C3H leukemia #19.*—This arose 4 years ago in a C3H male 10 months after it had received a transplant into the anterior chamber of the eye by a technique described previously (*2*). The transplant consisted of a fragment of thymus from a C3H embryo which had been dipped in an extract prepared from 6 pooled AKR leukemias [same 6 leukemias referred to in 1)]. Leukemia #19 is a lymphatic leukemia specific for C3H mice. It produces enlargement and hemorrhage of peripheral lymph nodes and enlargement of liver and spleen. Death occurs in 8 to 10 days in all 6- to 10-week-old C3H mice into which it is transplanted subcutaneously. It is at present in its 155th transplant generation.

3) *AKR leukemia #RIL6.*—This represents the above-mentioned 6 pooled AKR leukemias that were adapted to grow progressively in C3H mice by a method described in an earlier communication (*20*). It is a lymphatic leukemia and occasionally produces hemorrhagic lesions. This tumor was carried through the 94th transplant generation and grew in both AKR and C3H mice, with fatal outcome.

Besides the 3 leukemias described, 20 additional transplantable mouse tumors and 8 primary tumors have been tested for the presence of LCM virus. Of the transplantable tumors 4 were parotid-gland tumors and 16 were leukemias; of the primary tumors 3 were parotid-gland tumors and 5 were leukemias. Six of these 28 merit attention here, as LCM virus was recovered and identified in 4 and presumptive evidence of the presence of the virus was obtained in 2 others.

A brief description of these tumors follows: The 4 from which LCM virus was recovered are given first. In the last 2, an agent was demonstrated which produced a disease identical to LCM, but it was not identified by immunization tests.

1) *AK-n leukemia #60.*—This arose spontaneously in an AK-n mouse [kindly given to us by Dr. Ludwik Gross (*2*)] and is being carried as a transplanted leukemia in AKR mice. It is now in its 70th transplant generation.

2) *Subline C, G-153 of leukemia L1210.*—A transplantable leukemia that was carried in transfer, as an ascites tumor with 8-azaguanine (guanazolo) to the 98th transfer, and was obtained from Dr. L. W. Law, National Cancer Institute, Bethesda, Maryland.

3) *Subline Am D, FX of leukemia L1210.*—Law *et al.* (*22*) reported it as an amethopterin-dependent tumor. It was obtained from Dr. A. Goldin, National Cancer Institute, Bethesda, Maryland. At that time he had carried it in his laboratory for 5 transfer generations with amethopterin treatment.

The 2 L1210 sublines were derived from a leukemia that arose in strain DBA/2Lw mice (*21*).

4) *Primary parotid-gland tumor #2816.*—This arose in a (C3Hf × AKR)F_1 hybrid mouse 4 months of age that had been inoculated when newborn with a cell-free extract prepared from a paraganglioma. (The paraganglioma and the method for recovering the virus from the parotid-gland tumor will be described in a later publication.)

5) *Primary parotid-gland tumor #2929.*—This appeared in a 3½-month-old (C3Hf × AKR)F_1 female that had been inoculated when newborn with a cell-free extract prepared from the 49th transplant generation of AK-n leukemia #60 (referred to in paragraph 1).

6) *Transplantable parotid-gland tumor #818.*—This appeared in a 12-month-old C3H female that had been inoculated when newborn with a cell-free extract prepared from the spontaneous AK-n leukemia #60 (*see* paragraph 1).

Leukemias #1124, #19, #RIL6, and #60 were carried for 3 to 4 years as transplantable leukemias, and cell-free extracts prepared from them at different transplant generations have yielded a high percentage of neoplasms when inoculated into newborn mice (table 1). Cell-free preparations from the other tumors have not been tested for their tumor-inciting ability.

Procedures

During the initial stage of the work reported here, two methods were employed in searching for a virus in the tumors studied:

1) In the preliminary studies with leukemia #1124, the liver, spleen, lymph nodes, kidneys, and local tumors were removed from a mouse carrying the 35th transplant generation, homogenized in a Potter-Elvehjem homogenizer, and diluted to a 20 percent suspension with Ringer's lactate solution (Lilly). This suspension was centrifuged in a PR-1 International centrifuge at 3,000 r.p.m. (1,400× g) for 15 minutes at 0° C. The supernatant was removed and centrifuged at 9,500 r.p.m. (7,000× g) for 10 minutes at 0° C. Nine newborn C3H mice less than 12 hours old were inoculated intraperitoneally with 0.1 ml. of the clear supernatant from the second centrifugation.

Two moribund mice were sacrificed on the 15th day and the liver, spleen, lymph nodes, thymus, and kidneys were removed and prepared as above. The supernatant from the second centrifugation was filtered through a Morton glass filter which had been shown to hold back a culture of *Escherichia coli*.

Five 14- to 16-day-old fetuses were removed aseptically from a normal, pregnant C3H mouse. Their heads, legs, and tails were removed and the

TABLE 1.—*Tumors in mice inoculated when newborn with cell-free extracts prepared from 4 transplanted mouse leukemias**

Leukemia	Transplant generation	Mouse strain	No. of mice inoculated	No. of mice surviving 2 mos. or longer	Mice developing tumors at 2.5 to 12 months†						No. survivors / No. developing tumors
					Leuk.	Parotid	Sub-max.	Adrenal	Mamm.	Other organs	
C3H leukemia #1124	8	C3H	25	9	0	1	0	0	6‡	0	7/9
	20	C3H	20	0	0	0	0	0	0	0	0/0
C3H leukemia #19§	7	C3H	12	7	0	2	0	0	0	0	2/7
	15	C3H	10	3	0	0	0	0	0	0	0/3
	17	C3H	10	3	0	0	0	1	0	0	1/3
	18	C3Hf	16	3	0	1	0	1	0	0	2/3
	24	C3Hf	8	7	0	5	0	6	2	4	7/7
	33	C3H	9	2	0	0	0	1	0	0	1/2
	89	(C3Hf×AKR)F_1	47	20	0	6	0	0	0	0	6/20
AKR leukemia adapted to C3H mice (RIL6)§	29	C3H	8	6	0	3	0	0	0	0	3/6
	39	C3H	5	3	0	1	0	0	0	0	1/3
	51	C3H	7	4	3	0	0	0	0	1	4/4
AK-n #60§	Before transplantation	C3H	12	8	0	1	0	0	0	0	1/8
	9	(C3Hf×AKR)F_1	6	5	5	0	0	0	0	0	5/5
	11	(C3Hf×AKR)F_1	7	3	1	3	0	2	1	0	3/3
	30	(C3Hf×AKR)F_1	20	16	0	14	5	0	1	0	14/16
	47–49	(C3Hf×AKR)F_1	77	49	1	23	0	0	2	0	23/49

*Some of these data were taken from earlier publications (*2,5*).
†Many of the mice had multiple tumors; the pituitary gland did not appear involved in any of these.
‡These arose in mice carrying the mammary tumor agent.
§Mice inoculated with extracts of these leukemias where the entire litter died early are not included.

remainder was passed through a 20-gauge tumor press. The tissue mince was divided into two parts; to one was added sterile Ringer's lactate solution and to the other some of the filtrate described; each was stirred thoroughly. Only enough solution was added to enable the tissue mince to be taken up in a 2 ml. syringe. After the mixture had stood at room temperature for 15 minutes, 6 C3H males, 4 weeks of age, were inoculated subcutaneously with 0.3 ml. of the tissue mince plus filtrate, and 6 others with 0.3 ml. of tissue mince plus lactate solution; a third group of 6 was similarly inoculated with 0.3 ml. of filtrate only. Aseptic methods were used. Mice receiving tissue mince plus filtrate were used for passage (illness developed on the 6th day and deaths began on the 9th). Fifteen serial passages were made in the manner described. Attempts to carry the agent without tissue mince were unsuccessful. Cultures were made from the organs and tissue mince; no bacteria were ever found.

2) In early studies with leukemias #19, #RIL6, and #60, tissues were prepared as described above, but 4- to 6-week-old C3H mice which were to receive the filtrate-tissue mince were prepared either by X irradiation (250 r total-body irradiation[3]) about 2 hours before inoculating or by inoculation with cortisone (2 mg. for 3 successive days prior to injection of tissue preparation). Such mice developed a disease like that seen in mice inoculated with the filterable agent from leukemia #1124 and these were used for passage. The agent obtained from leukemias #19 and #60 was carried in pretreated mice; that from #RIL6, in untreated ones.

A titration of the agent from leukemia #1124 was carried out during its fifth serial passage. Liver, spleen, thymus, lymph nodes, and kidneys from a moribund mouse were homogenized, diluted 1:10 in Ringer's lactate solution and centrifuged at 3,000 r.p.m. at 0° C. for 15 minutes. The supernatant was used to make serial tenfold dilutions up to 1:100,000 in Ringer's lactate solution, and 0.25 ml. of each dilution was mixed with fresh tissue mince (approximately 0.6 gm. for each dilution). Then 0.2 to 0.25 ml. was inoculated subcutaneously into 5-week-old C3H mice, using 5 mice for each dilution. A dilution of 10^{-4} caused death in all mice inoculated, with 2 of the 5 mice surviving until the 30th day. At 10^{-5} there were no deaths.

In later stages of these investigations, the search for virus was made by intracerebral injection of extracts, prepared as described, into Swiss mice 4 to 6 weeks old, which had not been pretreated. When such a mouse became ill, its brain was removed, ground in a mortar, and suspended in buffered saline to make a dilution of 1:1,000. This material was injected intracerebrally into fresh Swiss mice and also into others which had been immunized previously against LCM by subcutaneous injection of a known strain of the virus (*23*). Identification of the agent from tumor filtrate was in each instance based upon 1) incubation period in intracerebrally injected mice, 2) nature of the illness produced, 3) failure to cause illness in specifically immunized mice, and 4) pathologic changes in prepared

[3] Physical factors for irradiation were: total-body irradiation 1 tube; 250 KVP, 30 μ; beam filtration, 1.0 mm. Cu + 1 : 0 Al target-mouse distance for tube, 50 cm.; dose rate 75 r per minute.

sections of tissues.[4] In one instance mice were injected subcutaneously with virus derived from a parotid-gland tumor (#2816) and challenged intracerebrally 2 weeks later with known LCM virus; all survived.

Results

Specific identification of virus was not accomplished on the tumors mentioned with the cell-free extracts used in the earlier studies. More recently, LCM virus has been recovered by the methods described from 4 mouse leukemias, #19, #60, L1210 guanazolo-dependent, and L1210 amethopterin-dependent. It was also recovered from 1 primary parotid-gland tumor, #2816, referred to previously. Results of the immunization experiments are given in tables 2 and 3.

Since the earlier material is no longer available, no proof of the presence of a known virus is possible. To assist in reasonable conjecture as to the

TABLE 2.—*Protection against virus recovered from mouse leukemias by specific immunization with LCM virus*

Virus isolated from:	Survival of mice given virus intracerebrally	
	Mice immune to LCM	Mice nonimmune to LCM
Leukemia #19	5/5	0/5
Leukemia #60	5/5	0/5
Leukemia L1210 (guanazolo-dependent)	5/5	0/5
Leukemia L1210 (amethopterin-dependent)	5/5	0/5
Parotid-gland tumor #2816	5/5	0/5

TABLE 3.—*Cross immunity between a known LCM virus and the virus recovered from a primary parotid-gland tumor (#2816)**

Immunologic status of mice	Post-inoculation day†	No. mice found dead	No. mice surviving 2 weeks or longer
Immunized 12 days previously by subcutaneously injecting virus obtained from parotid-gland tumor	<5	2	17
	11	1	
Immunized against known LCM virus 12 days previously			10
Not immune to LCM	6	7	0
	7	1	

*All mice challenged by intracerebral inoculation with 0.03 ml. 10^{-3} dilution of LCM virus.
†All mice were observed for 14 days following inoculation.

[4] We are indebted to Dr. Thelma Dunn and to Dr. L. N. Loomis, of the National Institutes of Health, for examination of the tissues. Their reports indicate findings consistent with LCM infection.

likelihood of the cell-free extracts containing LCM, the observations made at the time are given here.

Leukemia #1124.—First passage of the agent in 4-week-old strain C3H mice. Six mice received only cell-free filtrate (*see* p. 238) subcutaneously; none became ill. Six which received subcutaneous injections of filtrate plus fetal-tissue mince developed growths at the inoculation sites and died between the 6th and 9th days. Six receiving only fetal-tissue mince also developed growths at the inoculation sites but remained in good health. These were observed for 18 months.

Peripheral blood counts made on mice inoculated with filtrate-tissue mince generally showed granulocytosis, the counts ranging from 36,000 to 90,000 per mm.^{3} Occasionally the mice developed petechiae or ecchymotic areas which were demonstrable after shaving an area of skin; such mice had a drop in platelets to 20,000–30,000 per mm.^{3} Mice with petechiae and ecchymotic areas are shown in figures 1 and 2.

By the fifth passage the agent had increased in virulence, killing some mice as early as 5 days. These mice had a severe anemia with reticulocytosis, anisocytosis, and poikilocytosis. The hemoglobin often dropped to 4 gm. or below. Many of the mice that died early had a leukopenia of 3,000 to 4,000 cells per mm.^{3} Most of the inoculated mice lost weight.

Mice dying from the filtrate-tissue mince were found to have developed subcutaneous spreading, hemorrhagic, gelatinous outgrowths from the fetal mass. In some, these outgrowths spread throughout the subcutaneous tissues of the abdomen extending to the back, and appeared to penetrate the muscle wall of the abdomen. The peripheral lymph nodes were also hemorrhagic and, in some instances, definitely enlarged. The peritoneal and thoracic cavities were filled with a cloudy fluid which was free from bacterial contamination as shown by culture, but which showed great numbers of immature white blood cells of the granular type. In 1 mouse, 6 ml. of fluid were recovered from the peritoneal cavity. In later passages of the virus, fluid in the thoracic and peritoneal cavities was found only occasionally. In all these mice gross examination revealed a small thymus and spleen and a pale liver, but the lungs appeared uninvolved; in some, the adrenals appeared hemorrhagic. The parotid glands and pancreas often appeared edematous. Muscle tissue often had a gelatinous appearance.

Leukemias #19, #RIL6, and #60.—The filterable agents recovered from these leukemias produced the same lesion, as observed by histologic studies and by gross appearance, and possessed the same biological and physical characteristics as the filterable agent from leukemia #1124. All were highly lethal when inoculated into newborn strain C3H mice, often killing the entire litter. None were lethal when inoculated subcutaneously into 4- to 6-week-old C3H mice, but, when mixed with a fresh fetal-tissue mince and inoculated subcutaneously, they produced the fatal inflammatory disease described. (A laboratory strain of LCM virus inoculated subcutaneously into 4- to 6-week-old mice had no lethal effect and resulted only in immunizing the animal to the lethal effects of intracerebral

inoculation. However, when the LCM virus was added to mouse feta tissue and inoculated subcutaneously in the manner described, it produced a fatal inflammatory disease.)

These changes, produced by the cell-free extracts used in the earlier studies, cannot be taken to indicate the specific identity of the agent which caused them. They do not exclude LCM and, considered along with the established fact of the presence of this virus in tumor material obtained later, they would add partial support to the conjecture that the earlier extracts contained this virus.

In addition to the early observations and the isolation of the specific virus, later presumptive evidence of the presence of LCM virus in 2 other tumors has been obtained: 1) primary parotid-gland tumor #2929 and 2) transplanted parotid-gland tumor #818. Cell-free extracts from these 2 tumors caused a disease in mice indistinguishable from LCM.

Normal mice from stocks at the National Institutes of Health of strains C3H/He, DBA/2J, BALB/cAn, AKR, Swiss, and A/He were tested for possible LCM viremia, using a technique described elsewhere (*23*). No evidence of virus was obtained. None of the mice from these stocks was resistant to intracerebral challenge with LCM virus.

Summary

Cell-free extracts or filtrates prepared from 4 transplantable mouse leukemias were fatal for a high percentage of newborn mice inoculated subcutaneously or intraperitoneally. A virus-like agent was demonstrated in each of the 4 leukemias, but, although these agents were carried for up to 15 serial passages, they were not maintained for later identification. The agent had little or no effect when inoculated subcutaneously into 4- to 6-week-old mice; however, when it was added to a freshly prepared tissue mince (14- to 16-day mouse fetuses) and inoculated subcutaneously it caused death in 6 to 12 days. The newborn mice surviving the inoculation developed a significant incidence of parotid-gland tumors and other neoplasms.

Later attempts to identify the agent in 2 of these leukemias resulted in the recovery of LCM virus. This virus was also recovered from 2 other transplantable leukemias and from a primary parotid-gland tumor.

The source of LCM virus has not been demonstrated. All stock mice tested have been negative.

The significance of LCM virus in the leukemias which yielded cell-free preparations with tumor-inciting properties has not been established.

References

(*1*) STEWART, S. E.: Leukemia in mice produced by a filterable agent present in AKR leukemic tissues with notes on a sarcoma produced by the same agent. (Abstract.) Anat. Rec. 117: 532, 1953.

(*2*) ———: Neoplasms in mice inoculated with cell-free extracts of filtrates of leukemic mouse tissues. I. Neoplasms of the parotid and adrenal glands. J. Nat. Cancer Inst. 15: 1391–1415, 1955.

(3) STEWART, S. E.: Neoplasms in mice inoculated with cell-free extracts or filtrates of leukemic mouse tissues. II. Leukemia in hybrid mice produced by cell-free filtrates. J. Nat. Cancer Inst. 16: 41–53, 1955.

(4) ———: The propagation of a filterable agent which produces neoplasms of the parotid gland in mice. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 48, 1955.

(5) TAYLOR, M. J., and MACDOWELL, E. C.: Mouse leukemia. XIV. Freeing transplanted line I from a contaminating virus. Cancer Res. 9: 144–149, 1949.

(6) DEBRUYN, W. M.: A lymphopenia-causing agent, probably a virus, found in mice after injection with tumor tissue and with cell-free filtrates of lymphosarcoma T 86157 (MB). Cancer Res. 9: 395–397, 1949.

(7) LAW, L. W., and DUNN, T. B.: Effects of a filterable self-propagating contaminant on a transplantable acute lymphoid leukemia in mice. J. Nat. Cancer Inst. 11: 1037–1055, 1951.

(8) NELSON, J. B.: Acute hepatitis associated with mouse leukemia. I. Pathological features and transmission of the disease. J. Exper. Med. 96: 293–302, 1952.

(9) BRAUNSTEINER, H., and FRIEND, C.: Viral hepatitis associated with transplantable mouse leukemia. I. Acute hepatic manifestations following treatment with urethane or methylformamide. J. Exper. Med. 100: 665–674, 1954.

(10) LINDORFER, R. K., and SYVERTON, J. T.: The characterization of an unidentified virus found in association with line I leukemia. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 33–34, 1953.

(11) LEWIS, M. R.: Myeloid hyperplasia brought about in mice by growth of dibenzanthracene tumors and its relation to transplantability of tumors into mice of alien strains. Am. J. Cancer 29: 510–516, 1937.

(12) JAFFE, R. H.: Über die extramedullare Blutbildung bei anamischen Mausen. Beitr. path. Anat. 68: 224–257, 1921.

(13) HUEPER, W. C.: Leukemoid leukemic conditions in white mice with spontaneous mammary carcinoma. Folia haemat. 52: 167–178, 1934.

(14) MAVER, M. E., and DUNN, T. B.: Catheptic activities of neoplasms and of the tissues of normal and tumor-bearing mice correlated with histologic changes. J. Nat. Cancer Inst. 6: 49–60, 1945.

(15) BATEMAN, J. C.: Leukemoid reactions to transplanted mouse tumors. J. Nat. Cancer Inst. 11: 671–687, 1951.

(16) PARSONS, L. D.: Leukaemia coincident with and transmissible by a spindle-celled sarcoma in the mouse. J. Path. & Bact. 40: 45–54, 1935.

(17) ———: Blood changes in mice bearing experimental sarcomas. (A) Sarcomas induced by a derivative of 1:2:5:6 dibenzanthracene; (B) Sarcoma produced by cell-free filtrates of mal. sarcoma. I. J. Path & Bact. 43: 1–22, 1936.

(18) ———: Changes in the lymph glands of tumour-bearing mice. J. Path. & Bact. 47: 501–523, 1938.

(19) UPTON, A. G., and FURTH, J.: A transmissible disease of mice characterized by anemia, leukopenia, splenomegaly and myelosclerosis. Acta haemat. 13: 65–76, 1955.

(20) STEWART, S. E.: Leukemia homotransplants in mice. J. Nat. Cancer Inst. 14: 1187–1193, 1954.

(21) LAW, L. W., DUNN, T. B., BOYLE, P. J., and MILLER, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(22) LAW, L. W.: Response of a resistant variant of leukemic cells to an antagonist of pteroylglutamic acid. Proc. Soc. Exper. Biol. & Med. 77: 340–344, 1951.

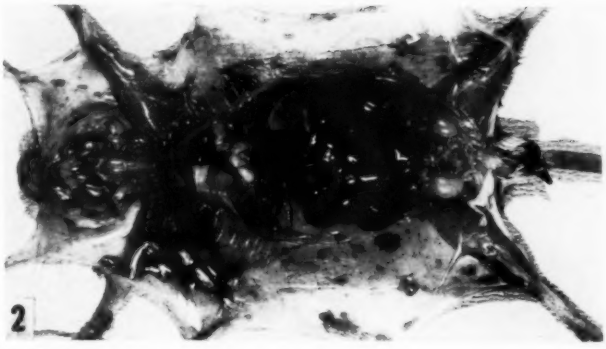
(23) HAAS, V. H.: Some relationships between lymphocytic choriomeningitis (LCM) virus and mice. J. Infect. Dis. 94: 187–198, 1954.

PLATE

PLATE 18

FIGURE 1.—Strain C3H female inoculated with 8th passage of agent from leukemia #1124.

FIGURE 2.—Strain C3H male inoculated with 10th passage of virus from leukemia #19.



383538—56——14

Announcement

Seventh International Cancer Congress

The International Union Against Cancer will hold the 7th International Cancer Congress in London on July 7 to 12, 1958. Because it is anticipated that transportation facilities will be greatly congested, it is essential that reservations be made as early as possible. To assure accommodations desired, reservations should be made by January 1957 for tourist-class steamship; by May 1957 for cabin-class steamship; by January 1958 for tourist-class plane; and by April 1958 for first-class plane.

U. S. A. National Committee on the
International Union Against Cancer
National Academy of Sciences
2101 Constitution Avenue, N. W.
Washington 25, D. C.

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited.

Published by the U. S. Department of Health, Education, and Welfare in accordance with section 220, title 44, U. S. Code

Approval by the Director of the Bureau of the Budget for the printing of this publication expires on 18 August 1957

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1956

For sale by the Superintendent of Documents, U. S. Government Printing Office
Washington 25, D. C. - Price per copy $2.00.

Subscription price for two volumes per year in the United States, Canada, and Mexico, $20.00; foreign, $25.00.

CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 17 • NO. 2 • AUGUST 1956

Page

The histogenesis of Rous sarcoma I induced by partially purified virus. *L. N. Loomis* and *A. W. Pratt* 101

Transplantability of a canine thyroid carcinoma through thirty generations in mixed-breed puppies. *M. W. Allam, L. S. Lombard, E. L. Stubbs,* and *J. F. Shirer* 123

Effect of glutamine on the growth and metabolism of liver cells *in vitro.* *B. B. Westfall, V. J. Evans, Ellison V. Peppers, Naomi M. Hawkins,* and *W. R. Earle* 131

Daily eosinophil rhythm in mice bearing a transplanted mammary carcinoma. *Franz Halberg, Halvor Vermund;* and *John J. Bittner* 139

Enzyme changes induced in normal and malignant tissues with chemical agents. VII. Effect on hydrolytic and synthetic enzymes of diphosphopyridine nucleotide in sarcoma 37. *V. S. Waravdekar, O. H. Powers,* and *J. Leiter* .. 145

The probability of developing cancer. *Irving D. Goldberg, Morton L. Levin, Paul R. Gerhardt, Vincent H. Handy,* and *Rita E. Cashman* 155

Digital computer studies of cell multiplication by Monte Carlo methods. *Joseph G. Hoffman, Nicholas Metropolis,* and *Verna Gardiner* 175

The metabolism of L-histidine-2-C^{14} in the normal and hepatoma-bearing rat. II. Protein and nucleic acid relationships. *James C. Reid, Odette S. Temmer,* and *Martha O. Bacon* 189

Modification of treatment schedules in the management of advanced mouse leukemia with amethopterin. *Abraham Goldin, John M. Venditti, Stewart R. Humphreys,* and *Nathan Mantel* 203

Urinary excretion of corticosteroids in guinea pigs with malignant neoplastic diseases. *Eli M. Nadel* and *Shlomo Burstein* 213

Effect of the virus of lymphocytic choriomeningitis on the course of leukemia in guinea pigs and mice. *Eli M. Nadel* and *Victor H. Haas* 221

Lymphocytic choriomeningitis virus in mouse neoplasms. *Sarah E. Stewart* and *Victor H. Haas* 233

Announcement:
Seventh International Cancer Congress 247